1931

Volume LXI 97 Number 2

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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OCTOBER, 1931

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between May 1 and November 1 and to the Zoological Laboratory, Harvard University, Cambridge, Mass., during the remainder of the year.

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

Vol. LXI, No. 2 October, 1931

THE

BIOLOGICAL BULLETIN

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CONTINUOUS VERSUS INTERRUPTED IRRADIATION AND THE RATE OF MUTATION IN DROSOPHILA

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It has been shown by Hanson and Heys (1928) for radium and by Oliver (1930) for X-rays that the rate of mutation in *Drosophila*, as measured by the percentage of sex-linked lethals produced, is determined by the strength of the dose used in the treatments. In general their results justify the conclusion that the rate is directly proportional to the dosage employed. This conclusion is based on results obtained in experiments in which the treatment was given continuously, and in which the only variable factor involved was a difference in the length of exposure. If all other factors are kept constant, the length of the treatment will determine the amount of ionizing radiation to which the flies are exposed. Under these conditions of experimentation they find, for example, that if the time of exposure is doubled, the number of sex-linked lethals produced will also be doubled.

It would seem to be a matter of interest to determine what effect on the mutation rate would follow if the dosage were given in fractions at regularly spaced intervals, instead of continuously as a single dose. Would the summation of the several spaced treatments give a cumulative effect on the rate of mutation, or would the rate be the same as for a single treatment in which the same total dosage was used?

The writer has carried out a series of experiments with a view to finding an answer to this question. In all, 16,963 F_2 cultures have been developed from the several different experiments performed. The method employed was the same as that used by Oliver (1930) and others. The treated flies were tested by Muller's CIB method, which has been fully described by several different writers. The chief point to emphasize is the one already mentioned by Oliver: not all F_2 female cultures are due to point mutation lethals; some result from chromosome abnormalities. Genetic tests were made for the detection of such cases, and those that were found have been excluded from the data listed in

the accompanying table. Four separate sets of experiments were carried out, and all of the treated and control P_1 males for a given set were taken from a single culture bottle. The treated flies belonged to a wild-type strain of *Drosophila melanogaster* that had been inbred for many generations, over a period of two years.

TABLE

Groups of P_1 Males	Type of Filter	Target Distance	Nature of Treatment	Time Interval	Total Time	Total Dose in r Units	Number of F_2 Tubes	Lethal Mutations	Percentage of Lethals
		cm.							
1....	alum.	12	continuous		16 min.	1654	971	49	4.95
2....	alum.	12	spaced	12 hr.	16 min.	1654	993	62	6.15
3....	alum.	12	spaced	6 hr.	16 min.	1654	981	71	7.14
4....	card	12	continuous		8 min.	2558	518	39	7.41
5....	card	12	spaced	12 hr.	8 min.	2558	345	45	12.95
6....	controls						986	1	0.10
7....	card	23	continuous		10 min.	1234	863	28	3.11
8....	card	23	spaced	24 hr.	10 min.±	1220	876	31	3.33
9....	card	23	spaced	12 hr.	10 min.±	1221	936	40	4.06
10....	card	23	spaced	8 hr.	10 min.±	1219	856	34	3.76
11....	card	23	spaced	1 hr.	10 min.±	1220	1014	32	2.94
12....	card	23	spaced	30 min.	10 min.±	1234	962	33	3.22
13....	alum.	12	continuous		8 min.	864	919	22	2.19
14....	alum.	12	spaced	1 min.	8 min.±	872	980	21	1.93
15....	controls						951	2	0.21
16....	alum.	3	continuous		12 hr.	radium	544	58	10.44
17....	alum.	3	spaced	12 hr.	12 hr.	radium	452	48	10.40
18....	controls						453	1	0.22

The first series of experiments, which was of a preliminary character, gave results that were not decisive, both because the numbers employed were too small, and because the controls proved to be inadequate. In these tests two groups of larvæ were X-rayed, one continuously and the other intermittently, and two groups of adult males were treated in the same manner. The intermittent exposures were given at regularly spaced intervals, and their sum in each instance was exactly equal to the time of the continuous exposure. The results showed that the groups given the spaced exposures yielded in the F_2 generations a higher percentage of sex-linked lethals than was found in the descendants of flies that had been given continuous treatments. The difference, however, was scarcely statistically significant. It was therefore decided to repeat the experiments on a larger scale, using longer treatments and varying the intervals in the spaced exposures.

In the second set of experiments six different groups of males (all from the same culture bottle) were used. Five of these were treated, and one was used as a control (Table, group 6). The first five groups were treated as follows: Group 1 was exposed for sixteen minutes with the machine operated at 50 kv., peak 10 ma., target distance 12 cm., and a 1-mm. aluminum filter. The total dosage in "r" units (column 7) was calculated from sample readings on a Victoreen dosimeter, taken just after the treatment had been completed. Group 2 was given sixteen exposures, each of exactly one-minute duration, at regular intervals of twelve hours. Group 3 was given thirty-two exposures, each of thirty seconds' duration, at regular intervals of six hours. Group 4 was treated continuously for eight minutes, with the machine operated at the same level as before, but with a card filter of .28 mm. thickness substituted for the aluminum filter. Finally, group 5 was given sixteen exposures, at regular intervals of twelve hours, and each of thirty seconds' duration.

The percentages (corrected for controls) of the tested sex-linked lethal mutations are shown in the last column of the table. It is obvious that groups 2 and 3 should be compared with group 1 and that group 5 should be compared with group 4. Calculations show that the difference between group 1 and group 2 is $.012 \pm .007$. The difference is less than twice its own probable error, and is therefore not significant. If we compare group 3 with group 1, the difference is found to be $.0219 \pm .0073$. Here the difference is exactly three times its own probable error and may or may not be significant. If groups 4 and 5 be compared in a similar manner, the difference is found to be $.0554 \pm .0138$, or slightly more than four times the probable error. It would therefore seem to be statistically significant.

The differences noted above may have been due to chance variations, or they may have resulted from experimental error, incident to starting and stopping the machine. If the sum of the several spaced exposures does not exactly equal (in the total amount of radiation) the time of the continuous exposure, differences in the mutation rate would necessarily occur. In giving the treatments every care was taken to make the two equal. The same voltage, milli-amperage, and target distance were used, and the intervals of time were carefully determined by means of a stop-watch. The total dosages (groups 1 to 5) were calculated, as stated above, from sample readings taken on the dosimeter just after the flies had been exposed, with the machine operated at the same level as was used in giving the treatments. But this method might be a source of error. It was therefore decided to arrange the apparatus so that the dosimeter readings could be taken at the time of making the exposures.

Numerous trial tests showed that so long as the machine was operated at the same level, one obtained very consistent readings.

The next series of experiments (groups 7 to 15) were conducted under the conditions just mentioned. The table shows the various details of the several experiments, except that the length of the individual exposures and the exact manner of calculating the total amounts of radiation as measured in *r* units is not revealed. With reference to the latter point, it may be stated that it was found necessary to vary slightly the length of the last exposure in the series of any given spaced test. This was done in order to make the total number of *r* units correspond as nearly as possible to that used in the continuous exposure. As an example, in group 7, the flies were exposed for ten minutes, and dosimeter readings were taken every thirty seconds. Calculations based on these twenty readings gave a total dosage of 1234 *r* units of radiation. The flies of group 8 were given eight exposures, each of one and a quarter minutes' duration, at regular intervals of twenty-four hours. Two readings were taken during each exposure, and after the first seven treatments had been completed the total dosage up to that point was determined. From the figure obtained, it was found necessary to shorten the last exposure five seconds, in order to treat this group of flies with a total dosage approximating that given to group 7. This gave a total of 1220 *r* units. The succeeding groups were handled in a similar manner. The deviation from the total time set by the continuous treatment was plus or minus about five seconds in each spaced test.

Groups 8 to 12 form a series in which the time interval for the spaced exposures was gradually shortened from twenty-four hours to thirty minutes. The corrected percentages of sex-linked lethal mutations run as follows: continuous (7), 3.11; twenty-four-hour interval (8), 3.33; twelve-hour interval (9), 4.06; eight-hour interval (10), 3.76; one-hour interval (11), 2.94; thirty-minute interval (12), 3.22. From this it will be seen that the percentages vary from 2.94 in group 11 to 4.06 in group 9. If one compares the percentage of lethals for the spaced exposures (groups 8 to 12) with that for the continuous exposure (group 7), one finds that the differences are not significant. The percentage of group 9 shows the widest divergency from that obtained in group 7 (3.11), but calculations show that the difference is $.0105 \pm .0060$, which is less than three times the probable error, and is therefore not statistically significant. Furthermore, the percentage of lethals obtained in the spaced exposures of group 11 is actually lower than that for group 7. Finally, in groups 13 and 14, in which there were eight-minute treatments, the spaced treatment again gave a lower percentage than was produced by the continuous exposure.

From the results obtained in this set of experiments, one may conclude that to give the treatment in fractional doses does not affect the rate of mutation in any way different from that found after a continuous exposure of equal strength had been used. The differences noted are due either to chance variation inherent in the material, or to small uncontrollable fluctuations in the voltage and milli-amperage of the machine. The ideal source of radiation for such a test would have been radium, because the amount of variation in dosage when this substance is used is negligible. Up to the time at which the above experiments were completed (July, 1930) radium was not available, but early last fall, through the kindness of Dr. W. C. Curtis of the National Research Council, 123 mg. of radium were loaned to the laboratory, and this was used in the following test (groups 16 and 17).

The arrangements for giving the radium treatments were such that it was possible to avoid any appreciable error in the time factor. Two groups of P_1 male flies were exposed; group 16 was given a continuous treatment lasting twelve hours, while group 17 was treated with twelve exposures each of exactly one hour, at regular intervals of twelve hours. The F_2 cultures gave for continuous and spaced treatments 10.44 per cent and 10.40 per cent of lethals, respectively. The difference is obviously insignificant.

Conclusion

The conclusion that one may draw from these experiments is that the rate of mutation, as measured by the number of sex-linked lethals produced, is the same whether the treatment is given in one dose or in several fractional doses. This is true so long as the total amount of radiation under each of the two conditions is made equal.

The effects of continuous and spaced radiation have been studied by several different investigators, but the only reference found, in which their effects on the rate of mutation are mentioned, is a paper by Serebrovsky and Dubinin (June, 1930), dealing with the production of mutations in *Drosophila* by X-rays. They report (p. 260) that continuous and interrupted exposures were given, and in this connection state that, " It is clear that lengthening the time of exposure with interruption or without raises the percent of mutation." One can not tell from this brief statement whether the percentages were the same or different under the two methods of treatment.

Most of the other papers concerned with methods of administering irradiation doses deal with embryological or histological materials, and they are therefore of no special interest in this connection. One of the latest papers along these lines is by F. G. Spear (May, 1931). Spear

used radium and studied and compared the delayed lethal effect on tissue cultures *in vitro*, after spaced and continuous irradiations had been given. He used the subcultivation method and concludes from his results that it is immaterial whether the irradiation is given in one or in several fractional doses—the delayed lethal effect is the same.

Austin, Texas,
June 1, 1931.

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GENETIC EVIDENCE FOR DIPLOIDISM OF BIPARENTAL MALES IN HABROBRACON

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I. Introduction

According to the theory of Dzierzon (1845), drones of the honeybee arise from unfertilized eggs. Within recent years this theory has been extended and developed into the general conception that males in Hymenoptera are haploid. It is unnecessary, here, to review the extensive literature dealing with this subject. Despite the evidence for parthenogenetic production of drones, many bee-breeders still insist that certain males in hybrid broods show paternal traits. If Dzierzon's theory is correct, how can this be possible?

The subject of sex determination in Hymenoptera assumes considerable interest to the geneticist from another point of view—that of the theory of genic balance as emphasized by Bridges (1925).

Investigations on the parasitic wasp *Habrobracon juglandis* (Ashmead) have served to clear our conceptions to some extent although much still remains to be done. Significant contributions already published may be briefly mentioned.

It was first shown in 1921 (Whiting, P. W., 1921) that certain males (patroclinous) in *Habrobracon,* contrary to expectation, occasionally show paternal traits and, therefore, do not arise from unfertilized eggs.

Subsequently, Anna R. Whiting found (1925) that such males were often abnormal and almost completely sterile.

The occurrence of new mutations made it possible to show (Whiting, Anna R., 1927) that these irregular males (biparental) resemble their sisters in that they inherit dominant traits from both parents.

It was at first supposed that they might be haploid mosaics. This idea was precluded (Whiting, Anna R., 1928) by the fact that, when the dominant members of two allelomorphic pairs affecting one and the same part of the body (the wings) were contributed by opposite parents, both dominant traits appeared. This indicates, though it does not prove, diploidism.

Still better evidence of diploidism of biparental males appeared (Whiting, Anna R., 1928) when a factor pair was used in which

dominance was reversed according to the structures affected. In the series of genes allelomorphic to orange (eye color), "ivory" has eyes and ocelli white while in "light" eyes are black and the ocelli, although of reduced pigmentation as compared with the normal black of wild type, are, nevertheless, dark brown in both males and females of pure "light" stock. Light-ivory compound females have eyes black and ocelli white, thereby exhibiting dominance of "light" in the former, of "ivory" in the latter. Their biparental brothers resemble them in this respect and thus demonstrate a duplex condition of this factor. Such a combination would be impossible in a haploid male.

Another indication of abnormal chromosomal constitution of biparental males is the fact that, occasionally, daughters that are almost completely sterile and often morphologically abnormal are produced (Whiting, Anna R., 1925).

The question as to why biparental males, if diploid, are, nevertheless, males still presents itself. It has been suggested that there may be a sex chromosome for which these males are simplex. The experiments summarized in the present paper were primarily planned for the purpose of locating such a chromosome by genetic test and, in addition, for studying the composition of daughters of biparental males.

The writer is indebted to Professor P. W. Whiting, at whose suggestion the investigation was begun, for guidance as the work progressed. Special thanks are due the co-workers on *Habrobracon* for supplying mutant stocks. Acknowledgment should be made in particular to the Committee on Effects of Radiation on Living Organisms (National Research Council) who, by a grant to Professor Whiting, have furnished technical help which has relieved the writer of much time-consuming labor and thus made possible a greater output of results.

II. Material and Methods

Since the purpose was to locate a sex-linked factor, new mutations were tested with this in mind. In most cases eye color (orange locus) was brought into the cross in such a way, with respect to the mutation, as to serve as an indicator of biparentalism; a male with a dominant eye color being crossed to a female with recessive eye color and the F_1 males with dominant eye color being taken as biparentals. It was not always possible to do this since no light eye colors are present in some of the stocks and, in cases thus far tested, related males and females must be crossed in order to obtain biparental males (Whiting, Anna R., 1925). In these stocks the dominant and recessive allelomorphs of the mutated locus had to be used alone.

The crosses may be divided into five main types involving: 1. nor-

mally inherited, recessive mutations in Lancasterized stocks; 2. recessive mutations in No. 11 and Minnesota stocks; 3. female sterile mutations; 4. interacting mutations; and 5. incompletely dominant or incompletely recessive mutations.

The triploid nature of the daughters of biparental males was tested by using some of these same mutations in a manner which will be described later.

III. Presentation of Data

A. Production of Biparental Males

1. Crosses Involving Normally Inherited, Recessive Mutations in Lancasterized Stocks.

Lancasterized stocks have all been graded up to a stock derived from a wild female taken in Lancaster, Pennsylvania in 1919. The method of inheritance by biparental males of certain normally inherited recessive mutations in these stocks has been previously discussed (Whiting, Anna R., 1927). It was shown that, when a female homozygous for the recessive mutant gene was crossed to a male carrying the normal allelomorph, there were produced not only recessive haploid sons but also a few sons with the dominant character. If females, homozygous for the normal allelomorph but marked by some other recessive factor (eye color, usually) were crossed to males, carrying the recessive mutant gene and the dominant marking factor, the biparental males showed only the dominant characters.

A series of quadruple allelomorphs affecting eye color (*O*, o^1, *o* and o^t) and three pairs of allelomorphs affecting the wings (*W*, *w*), (*R*, *r*) and (*D*, *d*) were shown to be inherited in the above manner (Whiting, Anna R., 1927).

The fact that of five black-eyed biparental males, obtained from crosses of orange wrinkled (*oowv*) females by type (*OW*) males, one had wrinkled wings, seemed to indicate something unusual about the inheritance of wrinkled wings. As shown in Table I this cross was repeated and 20 biparental males were obtained, none of which had wrinkled wings. The wrinkled wings of the above-mentioned male were no doubt, as they so often are, the result of accident of growth.

As shown further in Table I, three other loci were tested by reciprocal crosses. Long, *l*, affects both the wings and antennæ; eyeless, *el*, and small eye, k^s, or extremely small, k^e, (the latter 3 stocks kindly sent by Dr. Wilhelmina F. Dunning) affect the appearance of the eyes and shape of the head. All three factors were inherited by biparental males in the regular manner described above.

TABLE I

Crosses Involving Normally Inherited, Recessive Mutations in Lancasterized Stocks

Section	Parents ♀	Parents ♂	Matings	Progeny Biparental ♂♂	Progeny Impaternate ♂♂	Progeny ♀♀
1 a	*oowo*	+ (No. 1)	84	19	1219	2220
	oowo	*Y*	11	1	187	322
	oowo	+ (No. 31)	23	0	248	476
	oowo	*l*	11	0	109	208
	oodwo	+	3	5	43	93*
b	*oo*	*w*	50	73	2009	1313*
2 a	$o^io^il(L)l$	*Y*	7	2	87	92
	o^io^ill	*Y*	9	10	42	58
	o^io^ill	+ (No. 31)	2	0	9	11
b	o^io^i (No. 17)	*l*	6	2	68	28
	o^io^i	*l*	20	8	436	479
	cc	*l*	28	18	166	191
3 a	*elel*	+	20	1	203	743
	elel	*wa*	1	6	11	32
b	*oo* (No. 3)	*el*	6	5	164	178
4 a	$o^io^ik^ak^a$	+ (No. 1)	21	41	122	219
	$o^io^ik^ak^a$	*o* (No. 3)	3	3	28	31
b	k^ak^a	k^a	3	3	12	11
Total			308	197	5153	6705

* Taken from Table I—Whiting, Anna R., 1927.

2. Crosses Involving Recessive Mutations in No. 11 and Minnesota Stocks.

An attempt was made to obtain biparental males by crossing tapering, *ta*, (antennæ) males of Minnesota Yellow stock to orange-eyed females of No. 3 Lancaster stock, but none were obtained. Attempts have also been made to cross No. 11 individuals, Iowa City stock, to wasps from Lancaster stocks with the same results. Therefore, in dealing with mutant genes in these stocks, it was necessary to make the crosses between individuals of the same stock and to omit the eye color indicator. It is interesting to note that biparental males do occur when crosses are made within these stocks but do not with outcrossing, a result in agreement with previous findings for other stocks (Whiting, Anna R., 1925).

Table II gives a summary of the data obtained. Inheritance of three mutations: tapering, *ta*, (antennæ—Minnesota Yellow stock); wavy, *wa*, (wings—No. 11 stock) and semilong, *sl*, (antennæ and wings

TABLE II

Crosses Involving Recessive Mutations in No. 11 and Minnesota Stocks

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1	*oo* (No. 3).....	*ta* (Minn.)	10	0	260	431
	tata (Minn.).....	*My* (Minn.)	23	30	608	487
2	*wawa* (No. 11)....	+ (No. 11)	36		383	
	wawa (No. 11)....	+ (No. 11)	2	1	18	2
	elel (?).....	*wa* (No. 11)	1	6	11	32
3	*slsl* (No. 11).....	+ (No. 11)	17	64	205	184
Total			89	101	1485	1136

—No. 11 stock) was tested. The data show that wavy females usually produced only unfertilized eggs, though in many cases matings were observed. However, one biparental male was obtained from a wavy female. All three of these mutant genes were found to be inherited in the usual way by biparental males.

3. Crosses Involving Female Sterile Mutations.

Several of the mutant genes in *Habrobracon* seem to have a rather deleterious effect when present in the homozygous condition in the female. In many cases the female has to be given stung caterpillars upon which she is then able to feed and to oviposit, but such females are never as viable as type stock females.

When certain of the genes are present the deleterious effect becomes so great that the females are completely, or almost completely, sterile although the gonapophyses are of normal external appearance.

In dealing with these factors it was necessary to use heterozygous females and to assume that on the basis of chance some of the biparental males would carry the mutated gene. Haploid males with the mutant genes are fertile but usually have more or less difficulty in mating and, therefore, were rather difficult to test.

Table III gives a summary of data obtained from crosses involving four such factors. Fused, *f*, causes fusion of antennal and tarsal segments and an indentation near the apexes of the wings. Miniature, *m*, affects the whole wasps but especially wings and antennæ. Beaded, *b*,

TABLE III

Crosses Involving Female Sterile Mutations

Section	Parents ♀	Parents ♂	Matings	Progeny: Biparental ♂♂	Progeny: Impaternate ♂♂ Dominant	Progeny: Impaternate ♂♂ Recessive	Progeny: ♀♀
1 a	*oo'Ff*.....	+ (No. 1)	33	33	308	269	451
	oo'Ff.....	*f*	15	19	276	277	210†
b	*o'o'rr*.....	*f*	4	8	124		63
	oo (No. 3)...	*f*	1	2	1		6
2 a	*o'o'Mm*.....	*l*	21	8	279	173	503
	Oo'Mm.....	*o* (No. 3)	7	0	25	7	144
b	*o'o'rr*.....	*m*	12	0	99		219
	oo.....	*m*	1	2	14		33
	o'o'.....	*om*	3	3	63		32
3 a	*o'o'Bb*.....	+ (No. 1)	12	6	63	20	181
b	*oo*.....	*b*	14	0	221		229
	oo (No. 3)...	*b*		2			
4 b	*clcl*.....	*sp*	4	0	24		132
	oo (No. 3)...	*sp*	10	5	128		247
Total			137	88	1625	746	2450

* Classified as dominant or recessive according to factors *f*, *m*, *b*, *sp* for Sections 1, 2, 3, 4 respectively.

† 114 of these were fused, *ff*.

causes swelling of the leg segments. Spread, *sp*, causes the wings to be held down and spread out at the sides and produces a light spot on each side of the thorax.

Biparental males inherited these mutant genes in the usual way.

4. Crosses Involving Interacting Mutations.

Mutations at the three loci (orange, cantaloup and maroon) affecting eye color are, as one would expect, complementary to one another in the effect which they have upon eye color.

Crosses were made (Table IV) in such ways that the dominant of one locus was brought in with the recessive of another, by one parent while the allelomorphs were brought in by the other parent. For example, ivory females ($o^i o^i CC$) were crossed with cantaloup (Oc) males. In addition to ivory-eyed males and black-eyed females, 25 black-eyed males were obtained. These biparental males, like their sisters, received the dominant allelomorph to cantaloup from their mother, the

TABLE IV

Crosses Involving Interacting Mutations

Section	Parents ♀	Parents ♂	Matings	Progeny: Biparental ♂♂	Progeny: Impaternate ♂♂	Progeny: ♀♀
1 a	*cc*	*o*	53	1	273	698
	cc	+	28	18	166	191
b	$o^i o^i$	*c*	10	25	106	73
	oo	*c*	35	17	305	399
2 a	*mama*	*o*	9	11	52	140
	mama	+ (No. 1)	8	2	35	53
b	*oo* (No. 3)	*ma*	20	36	431	340
Total			163	110	1368	1894

dominant allelomorph to ivory from their father. These complementary dominant factors produced black eye color.

Reciprocal crosses involving the two loci orange and cantaloup and other crosses involving the two loci orange and maroon, were made. In all cases biparental males showed, by having type (black) eyes, that both dominant factors were present in their chromosomes.

5. Crosses Involving Incompletely Dominant or Incompletely Recessive Mutations.

An example of a factor pair with dominance reversed according to structure affected was cited in the introduction. Biparental males were found (Whiting, Anna R., 1928) to resemble their sisters in showing dominance of "ivory" in ocelli but of "light" in eyes, thereby demonstrating the presence of the two allelomorphs "light" (ocelli) and "ivory" (eyes) at the orange locus.

Inheritance of four other genes: dahlia, o^d, (eyes); stumpy, *st*,

(legs); yellow, *Y*, (antennæ) and short, *sh*, (wings) which produce distinct heterozygous types, has been studied (Table V).

Dahlia, o^d, is one of the quintuple allelomorphs (O, o^l, o^d, o, o^i) of the orange locus. It was noted that dahlia-orange (o^d-o) and, more

TABLE V

Crosses Involving Incompletely Dominant or Incompletely Recessive Mutations

Section	Parents		Matings	Progeny		
	♀	♂		Biparental ♂♂	Impaternate ♂♂	♀♀
1	*oo* (No. 3).....	o^d	17	10	112	118
	o^io^i.....	o^d	37	69	1080	741
2	o^io^i*stst*.....	*o* (No. 3)	10	2	27	45
	o^io^i*stst*.....	+ (No. 1)	7	0	21	39
3 a	o^io^i*YY*.....	*o* (No. 3)	10	0	38	89
	o^io^i(*o*)*YY*.....	+ (No. 1)	41	93	599	833
b	*oo* (No. 3).....	*Y*	13	1	190	319
	o^io^i (No. 17).....	*Y*	84	70	801	950
4 a	*ooshsh*.....	+ (No. 1)	171	81	3127	3572
	ooshsh.....	+ (No. 11)	4	0	74	42
b (1)	o^io^i (No. 17).....	*osh*	26	7	360	321
(2)	o^io^i (No. 17).....	*osh*	32	0	332	271
(3)	o^io^i (No. 17).....	*osh*	40	52	730	587
Totals			492	385	7491	7927

especially, dahlia-ivory (o^d-o^i) compound females were much lighter than homozygous dahlia females.

Crosses such that dahlia-ivory (o^d-o^i) and dahlia-orange (o^d-o) compound biparental males would be obtained, if possible, were made (Table V). Seventy-nine males were found which closely resembled their sisters, the compound (o^d-o and o^d-o^i) females, and were strikingly different from ivory or dahlia or orange haploid males. It seems fair to assume, since they also bred like biparental males, that these males must have been heterozygous and, therefore, duplex for eye color.

Eye color was always used as an indicator of male biparentalism in dealing with the other genes which affect variable traits.

The mutant gene stumpy, *st*, (legs) is an incomplete recessive. Stumpy wasps have extreme crowding and irregularity of tarsal segments so that, unless close examination is made, the legs appear to end

with the tibiæ. Heterozygous females show tarsal segments of approximately normal length but irregularly set together. The majority of these females, moreover, possess a new structure (an added spur on the prothoracic metatarsi resembling the prothoracic tibial spur) never found on type wasps.

Two biparental males were obtained (Table V) from crosses of stumpy females by type males. Both these males had the metatarsal spurs on both prothoracic legs and must, therefore, have been heterozygous for stumpy.

The factor yellow, *Y*, (antennæ) when wasps are reared under standard conditions (30° C.), changes the color of the three basal segments of the antennæ from the normal black to a clear yellow. The character is dependent for its typical expression upon this constant high temperature.

Reciprocal crosses were made (Table V). Wasps of those counts, only, in which biparental males appeared were preserved in alcohol as it was considered that these were reared under the most similar conditions. Later the preserved wasps were graded according to the scheme (devised by Lysbeth Hamilton Benkert) shown in Table VI.

TABLE VI

Frequency Distribution of Wasps of Various Genetic Compositions According to Grade of Yellow (Antennae)

Experiment No.	Genetic Composition	Class Values *							Mean Value
		1	1.5	2	2.5	3	4	5	
I	$Oo^{i}Yy$ ♀	42		36		59	16	18	2.6
	$Oo^{i}Yy$ ♂	17		15		17			2.0
	$o^{i}y$ ♂	120		40		1(?)			1.2
II	$Oo^{i}(o)Yy$ ♀			64	85	21			2.4
	$Oo^{i}(o)Yy$ ♂	4	23	50		5			1.9
	oY ♂			9		24			2.7
	oY ♂			32		62			2.7

Scheme of grading devised by Lysbeth Hamilton Benkert.
* 1—joints all dark.
2—3 joints yellow, slightly sooty.
3—3 joints clear yellow.
4—3½ joints clear yellow.
5—4 joints clear yellow.

The data show clearly that the biparental males are intermediate between the two types of haploid males (type and mutant) with respect to this character. The biparental males also differ somewhat from their

heterozygous sisters. Since females are normally lighter than males of the same stock this difference between heterozygous females and heterozygous males may be explained as a secondary sexual trait.

Variation in the character short, *sh*, (wings) depends upon multiple factors but there is, evidently, one main genic difference distinguishing short stock from type. Short overlaps with normal, especially at lower temperatures. Higher temperatures during development increase the difference from type.

In Experiment I orange short (No. 28) females were crossed to type (No. 1) males and black-eyed males were selected as biparentals. To make it possible to recognize biparental males on the basis of eye color in the reciprocal cross, it was necessary to use an ivory (recessive to orange) stock (No. 17) instead of the black (dominant to orange) stock (No. 1).

Since short is such a variable character, it was considered necessary to make measurements of the wings and to treat the data statistically. When biparental males were obtained, these and equal numbers of brothers and sisters from the same count were preserved in 95 per cent alcohol. Later the right mesothoracic wing and the head of each wasp were removed and placed on a slide. The parts were covered with separate pieces of cover glass and kept moistened with alcohol. Camera lucida measurements were made (magnification 50 ×) of the costal margin of the wing from the tip of the tegula to the end of the radius (point of fusion of R_1 and R_2). This may be called costal length, *c.l.* Similar measurements were taken of head width at the widest point including the eyes, *h.w.* The mean ratio of costal length to head width, *c.l./h.w.*, was used as a basis for comparing the various types. Table VII presents a list of the calculated means and mean differences.

Data given under Experiment I (Table VII) have been previously discussed (Torvik, Magnhild M., 1929). The black males, though they came from mothers homozygous for short, showed a significantly (4.23 × S.E.) greater mean *c.l./h.w.* ratio than that of their short brothers, demonstrating the presence of the type gene (*Sh*) in their chromosomes. Wings of females average somewhat larger than wings of males of the same stock. The mean *c.l./h.w.* ratio of the heterozygous females (*Sh sh*) was greater (6.34 × S.E.) than that of their biparental brothers, showing that these males must also be carrying short (*sh*). They must, therefore, be duplex for this factor (*Sh sh*).

Lower mean *c.l./h.w.* ratios were obtained for both biparental males and heterozygous females of Experiment II than for those of Experiment I. This difference indicates that short is a multiple factor character and that stocks No. 1 and No. 17 differ with respect to some of the factors.

In Experiment II wasps were first reared at the temperature of the former experiment (26°–27° C.) (Table V, line 1). Later an attempt was made to start the young at this temperature and then to shift the larvæ to another incubator running at a higher temperature (35° C.). As seen in Table V, line (2), from 32 crosses no biparental males were obtained and many dead larvæ were observed in vials thus transferred.

The cross was repeated at Woods Hole during the summer of 1929 and wasps which were later measured were obtained (Table V, line 3). The incubator at Woods Hole was kept at 30° C. rather than 26°–27° C. Also, these wasps were reared during the summer, when taking the vials from the incubator to transfer the mothers would have a less cooling effect upon the larvæ than the same treatment of larvæ reared during the winter. These facts help to explain the differences in mean ratios of wasps of Experiment I and Experiment II.

TABLE VII

Mean Ratios, Costal Length to Head Width, for Wasps of Various Genetic Compositions

Exp. No.	Genetic Composition	Group	$\frac{c.l.}{h.w.} \pm S.E.$	Group Differences	
I	*OoShsh* ♀	*a*	3.551 ± 0.025	*a–b*	0.215 ± 0.030
	OoShsh ♂	*b*	3.336 ± 0.023	*b–c*	0.121 ± 0.028
	osh ♂	*c*	3.215 ± 0.017		
II	*ooshsh* ♀	*d*	3.344 ± 0.019	*d–g*	0.158 ± 0.026
	$o^i o^i ShSh$ ♀	*e*	3.590 ± 0.020	*e–g*	0.404 ± 0.028
	$oo^i Shsh$ ♀	*f*	3.439 ± 0.014	*f–g*	0.253 ± 0.023
	$oo^i Shsh$ ♂	*g*	3.186 ± 0.018	*h–g*	0.259 ± 0.023
	$o^i Sh$ ♂	*h*	3.445 ± 0.014	*f–d*	0.095 ± 0.024
				e–f	0.151 ± 0.024
III*	*OSh* (No. 1)		3.566 ± 0.018	*h–f*	0.006 ± 0.019
	osh		3.182 ± 0.018	*e–d*	0.246 ± 0.026
				e–h	0.145 ± 0.024
				c–g	0.029 ± 0.025
				h–b	0.109 ± 0.027

* Data collected by Dorothy A. Binns.

In addition to the measurements of the three sorts of offspring—ivory males, orange males and orange females—of Experiment II, homozygous orange short (No. 28) females and ivory (No. 17) females were also measured. An interesting fact was brought out by these measurements (Table VII), namely, that the *c.l./h.w.* ratio is always greater for the female than for the same type of male. Heads of stock No. 17 males and females were approximately the same average width (male head 0.014 cm. narrower by camera lucida measure at × 50), but the male wing was shorter than the female wing (0.352 cm.). For this

reason the male ratio of *c.l./h.w.* would be proportionately lower than that of the female.

This point must be borne in mind in interpreting the data. It accounts for the fact that there is no significant difference between the mean *c.l./h.w.* ratio of heterozygous females and of No. 17 (type wings) males while the mean *c.l./h.w.* ratio of the heterozygous, biparental males is very much lower than either. The latter ratio is lower than the mean *c.l./h.w.* ratio of the heterozygous females, presumably of the same composition, because comparison is being made between males and females. It is lower than that of the No. 17 males because they are carrying only the gene for normal wings, while the biparental males show in this way that they are also carrying the gene for short wings.

The data also indicate (Table VII) that short is more dominant than recessive. The heterozygous females are more like the short females than like the No. 17 females (mean differences $4 \times$ S.E. and $6 \times$ S.E.).

It was thought that the mean *c.l./h.w.* ratio for short males from experiment I data supplemented by the same ratio for short females from stocks used for experiment II could be used in making comparisons with that ratio for biparental males of experiment II. Since the small change in temperature appears to have had an unexpectedly pronounced effect, and since male and female ratios are so different, these comparisons can not well be made. The biparental males of Experiment II are, however, very significantly different from their haploid (type wings) brothers with respect to mean *c.l./h.w.* ratio (Difference $-11 \times$ S.E.). It is, thereby, clearly demonstrated that short was inherited from the father by these biparental males.

Data supplementary to that of Experiment I were obtained from Dorothy A. Binns (Table VII, Ib). The wasps measured by her were reared under the same conditions as those of Experiment I.

The conclusion that biparental males are heterozygous for short seems justified for three reasons. 1. In Experiment I biparental males were shown to inherit short from their mother and in Experiment II they were definitely shown to inherit it from their father. 2. The mean *c.l./h.w.* ratio for biparental males of Experiment I is significantly different ($4 \times$ S.E.) from that of their short brothers and is also significantly different ($4 \times$ S.E.) from that of the type (wings) males (No. 17) of Experiment II. The difference from that of the type males (No. 1) of Experiment I would be even greater since No. 17 probably carries minor factors for short. 3. On the basis of supplementary data (Table VII, Ib) biparental males of Experiment I are shown to be intermediate between and markedly different from the two types of haploid males (normal and mutant) in mean *c.l./h.w.* ratio.

It may then be concluded from this study of inheritance of four factors determining distinguishable heterozygous types that biparental males are duplex for these factors.

B. Tests of Biparental Males

One of the strongest proofs of the diploidy of biparental males is the manner in which they breed. It was previously shown (Whiting, Anna R., 1927 and 1928) that over seventy-five per cent of biparental males were entirely sterile and that the remainder had produced a very small number of daughters which showed, with but rare exceptions, the dominant traits of the male parent. A few exceptional cases were reported early in the work on *Habrobracon* (Whiting, P. W., 1921). Seven biparental males with black eyes produced daughters with orange eyes; these males, therefore, bred like mosaics. Another exceptional male produced a fertile daughter with recessive eye color (Whiting, Anna R., 1927). As suggested, this may have been a thelytokous daughter of the female used for the test. One exceptional male was found (Whiting, Anna R., 1927) to breed as a heterozygote.

Table VIII gives a summary of the tests of biparental males made in connection with this study. Whenever possible, males from each type of cross were tested by mating them to females homozygous for the recessive factor involved. Individual males were sometimes mated to different females on successive occasions. Since this was in part a test of sterility, matings were always observed.

The data show that 90 per cent of the biparental males tested were sterile. Occasionally, one of these males had abnormal abdominal sclerites, abnormal legs, wings or antennæ but most of them appeared to be normal and all of them mated with females. There must, therefore, have been something abnormal about the spermatozoa or spermatogenesis of these males.

On the basis of tests here reported (Table VIII) we would expect one out of ten biparental males to produce daughters. Only one daughter was obtained from 90 matings for which 34 biparental (*Sh sh*) males were used (Table VIII). Sixteen daughters were produced by 5 of the 12 biparental (*Ta ta*) males tested. Only 17 matings of *Ta ta* males were observed and of these six were fertile. Four biparental *Sl sl* males, out of 20 tested, were fertile. Fertility of biparental males seems to differ with the factor involved and perhaps with the stock, though this point has not been tested particularly and is merely indicated by the above figures.

TABLE VIII

Tests of Biparental Males

Composition of Males	No. of Males Tested		No. of Matings		Offspring	
	Total	Fertile	Total	Fertile	♀♀	♂♂
WwOo	17	2	33	2	2	862
LlOo	9	1	9	1	1	153
Elel	5	0	5	0	0	503
K(k^s)k^s*O*(*o*)o^s *	14	3	16	3	18	1189
Tata	12	5	17	6	16	1184
Wawa	3	0	3	0	0	173
Slsl	20	4	35	4	25	1264
FfO(*o*)o^s *	12	0	17	0	0	687
MmOo	2	0	2	0	0	47
BbOo	2	0	3	0	0	134
SpspOo	1	0	1	0	0	2
CcOo(*O*) *	22	4	29	5	17	904
MamaOo(*O*) *	16	1	28	1	1	373
$o^d o^s$	14	3	16	3	5	677
Ststoo^s	2	0	5	0	0	517
YyO(*o*)o^s *	38	2	56	2	5	2586
ShshO(o^s)*o* *	34	1	90	1	1	2685
Totals	223	26	365	28	91	13934

* Symbols set in parentheses may be substituted for immediately preceding symbols.

C. Genetic Composition of Daughters of Biparental Males

Daughters of biparental males were found (Whiting, Anna R., 1925 and 1927) in previous experiments to be more nearly sterile and more abnormal than the biparental males. Certain of the 91 females obtained during the course of this investigation were also somewhat abnormal. Some of them had wrinkled wings or abnormal antennæ or irregular abdominal sclerites and a few had irregular gonapophyses, but only 7 were not tested with caterpillars. Seven died without stinging the caterpillars. Stung caterpillars were used with most of the others. Of the 77 thus tested 66 fed on the caterpillars but laid no eggs, 7 laid eggs which dried up without developing and 3 produced offspring.

The 3 fertile daughters, type in appearance, all came from one of the *Ta ta* males mentioned above. One daughter was produced in vial "*a*" (the first vial) by a heterozygous *Ta ta* female which ran through "*d*" (the fourth vial), producing also 58 males. This fertile daughter produced one type daughter which bred as a heterozygote, giving 10 tapering and 11 type males. From a second mating of this male with a tapering female there were obtained in "*a*" 18 tapering males and 2

sterile type females, in "*b*" 24 tapering males and 2 fertile type females and in "*c*" 13 tapering males. One of the fertile females produced one tapering male, the other produced 4 tapering males and 3 tapering females.

Since stung caterpillars were used with the tapering female of the second mating and for testing the daughters, contamination may explain the results. However, no cases of contamination were noted in other tests and 13 other daughters of *Ta ta* males were tested and found sterile.

The daughters of biparental males, in almost all cases, show only the dominant traits of their male parent regardless of how these traits entered his composition. In many cases one dominant was contributed by the male parent and another by the female parent of the biparental male and the daughter showed both of the traits.

It was interesting to note the appearance of characters determined by factors showing incomplete dominance. Daughters were obtained from o^d-o^i males mated to O-o^i and o^io^i females. The eye color of these daughters was about the same as that of biparental males or heterozygous females (o^d-o^i). Two *Yy* males produced daughters. One male was very dark; it was mated to a type (antennæ) female and produced 4 daughters with rather dark antennæ. The other was lighter; it was mated to a *Yy* female and produced a daughter having the 3 basal antennal segments clear yellow.

The small eye locus gave interesting results. Eighteen daughters were obtained from 3 matings. In 2 cases Kk^s males were mated to type females and produced 14 daughters, of which 4 had eyes much smaller than normal. In the other case, a Kk^s male was mated to a small eye female and the 4 daughters resulting had small eyes—much smaller than normal though not as small as small eye often is. This locus needs to be tested further.

For other factors (Table VIII), where dominance is known to exist, the daughters always showed the dominant traits of their male parent though he must also have carried recessives. These males, then, do not behave like diploid individuals; they do not segregate recessives. In order to explain this discrepancy it has been suggested that the sperm may be diploid.

If the sperm of biparental males are diploid, their daughters may be expected to be triploid. They must possess some irregularity in chromosomal constitution since they are often morphologically abnormal and rarely produce offspring.

The triploid nature of these daughters has been tested by means of sets of three pairs of factors, each dominant being brought in by a different individual. Two sets of such factors have been used.

In one instance the complementary eye color factors orange and cantaloup were used along with the factor for reduced wings. Seventeen biparental males resulted from the union of egg (*oCr*) with sperm (*Ocr*). These males had type (black) eyes and reduced wings. They (*OoCcrr*) were mated to orange cantaloup females (*ooccRR*). The 16 daughters from this cross were type showing the three dominants (*OCR*). This indicates probable triploidism.

More conclusive proof of the triploidy of the daughters was obtained by means of two linked (*Cc* and *Ll*) and one independently segregating (*Rr*) pairs of factors. Cantaloup (eye color) and long (antennæ, wings and legs) are linked and have a cross-over value of about ten per cent. Eighteen biparental males were obtained from crosses of cantaloup reduced (*cL/cL r/r*) females by long reduced (*Cl r*) males. The biparental males were type except for reduced wings and must, therefore, have been duplex for the *cl* chromosome. These reduced males *cL/Cl r/r*) were mated with cantaloup long (*cl/cl R/R*) females. One daughter was obtained. She has been thoroughly examined and compared with long and reduced wasps. One of her primary wings was not completely expanded, but camera lucida drawings were made of the other and of her antennæ and legs. These were compared with drawings of long. She was found to be type with respect to all three structures, showing that she possessed the dominant factor *L*. She was obviously non-reduced, *R*, and non-cantaloup (black-eyed), *C*, and hence must have resulted from union of sperm (*cL/Cl r/r*) with egg (*cl R*) and would, therefore, be (*cl/cL/Cl R/r/r*) triploid.

Since daughters of biparental males are often morphologically abnormal, rarely produce offspring and will inherit from three individuals in the manner shown above, it seems probable that they are triploid.

IV. Discussion

Data thus far obtained suggest that biparental males are duplex for all chromosomes studied. None of the seventeen mutations tested gave evidence of being simplex in biparental males and therefore located in a sex chromosome. Genetically we have as yet no sex chromosome and the question as to why biparental males are males is still unanswered.

Sex in *Drosophila* has been most conclusively shown to be dependent upon genic balance. Even the haploid has recently been shown (Bridges, C. B., 1930) to be female.

However, the situation in *Drosophila* must differ somehow from that in certain other forms where males are produced through haploid parthenogenesis and females are diploid. This apparently is true of most Hymenoptera (Whiting, P. W., 1918) and has been definitely

shown to be true of many coccids. In *Icerya purchasi* it was found (Schrader, Franz and Hughes-Schrader, Sally, 1926) that, "the individual chromosomes of the haploid set as found in the cells of the males correspond in size to the individual chromosomes of the diploid,—one member of each of the two morphologically distinct pairs of the diploid set apparently being present in the haploid group." The same is true of other coccids (Hughes-Schrader, Sally, 1930).

Sex in such forms can hardly be based on the same sort of genic balance as in *Drosophila*. Bridges (1925) suggested that, "at present the difference between haploid and diploid sexes must be referred to the same type of determination as that responsible for the larger size, rougher texture of eyes and other slight changes that distinguish the 3 N from the 2 N individual," in *Drosophila*.

Castle (1930) postulates that there is no minus sex-tendency in species which have haploid males. "The egg is homozygous for plus sex tendency (XX); the haploid male transmits in its one class of sperm this same sex-tendency (X)." He is unable to state why these haploid individuals, genetically female, are phenotypically male.

One interesting point is the fact that there has been no indication of intersexuality in *Habrobracon juglandis* (Ashmead) though cases of intersexuality were noted in *H. brevicornis* (Wesmael) (Whiting, P. W., and Whiting, Anna R., 1927, p. 112). The more biparental males are studied, the more closely is their genetic composition found to parallel that of the diploid female. In spite of this genetic similarity to females the biparental males are as definitely male in morphology and reactions as are haploid males nor does their added chromatin make them noticeably larger than haploid males.

V. Summary and Conclusions

1. Biparental males inherited dominant allelomorphs, of eleven pairs of factors showing ordinary dominance and recessiveness, from both parents when reciprocal crosses could be made or from either parent carrying the dominant when they could not.

2. Biparental males inherited complementary dominant factors, one from each parent, in such a way as to reconstitute the type character.

3. Homologous chromosomes were shown to be present in biparental males by means of linked factors.

4. Four factor pairs which produce distinct heterozygous types were inherited by biparental males in such a way as to demonstrate a duplex condition in each case.

5. Daughters of biparental males were shown to inherit, in almost all cases, only the dominant traits of the male parent regardless of how these traits entered his composition.

6. The triploid nature of these daughters and the diploid nature of the sperm of biparental males were tested by means of three pairs of factors, each dominant being brought in by a different individual.

7. Biparental males appear to be diploid and their daughters appear to be triploid.

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THE CHROMOSOMES OF THE DOMESTIC TURKEY

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In a previous article (Biological Bulletin, Vol. LII, No. 5, May, 1927), I have described the chromosomes of the Indian runner duck, giving the probable number and forms, and have proposed a scheme for sex-linkage and sex-determination. The present study was undertaken in 1927 to find out whether or not the conditions found in the duck exist in other avian forms.

This study is based on the examination of approximately 800 mitotic figures taken from 35 individuals. The same four general methods of technique were employed as in the former work, except that in the technique for sectioned material of the testis, Bergamot oil was used instead of cedar oil. Since the tissues of the turkey were more difficult to prepare than those of the duck, the utmost precision was necessary in order to obtain desired results.

As in the duck, the cells of the male contain an even number of chromosomes, and the cells of the female an odd number and one more than is found in the cells of the male. The number of chromosomes in the turkey appears to be the same as in the duck.

In the embryonic tissues of the male one cell was found which appeared to have 66 chromosomes (Fig. 2), but a large majority of the cells examined contained 76 chromosomes. In the embryonic tissues of the female two cells were found which contained 55 chromosomes each. One of these is shown in Fig. 7. The remainder of the female cells examined appeared to contain 77 chromosomes each. It would appear that the typical somatic numbers are 76 for the male and 77 for the female.

As in the duck the chromosomes are of three forms: J-shaped, rod-shaped, and globe-shaped, and as in the duck the chromosomes fall into three general groups. In the male of the turkey these groups consist of 6 pairs of large chromosomes, 3 pairs of short rod-shaped chromosomes, and 29 pairs of globe-shaped chromosomes. In the female there are 6 pairs of large chromosomes, resembling in size and form those of the male, plus one odd chromosome which is the largest in the group; 3 pairs of short rod-shaped chromosomes; and 29 pairs of globe-shaped chromosomes. It is apparent that the difference in the chromosomal

grouping in the duck and in the turkey is in the second and in the third groups. In the duck the second group contains nine pairs of short rod-shaped chromosomes and the third group contains 23 pairs of globe-shaped chromosomes.

In the aberrent cells of the male which contain less than 77 chromosomes (Fig. 2) all of the twelve large chromosomes of the first group are present, also the six short rod-shaped chromosomes of the second group. The missing chromosomes are the ten smallest of the third group. The same thing seems to be true in the aberrent cells of the female of less than 77 chromosomes. In these are present the 13 large chromosomes of the first group, the six rod-shaped chromosomes of the second group; but 22 of the smallest chromosomes of the third group are not present.

The same pliancy is noted in the chromosomes of the turkey as was evinced in the chromosomes of the duck. Because of this the chromo-

EXPLANATION OF PLATES

All figures from the turkey are reproduced at the same scale. The drawings were outlined with an Abbe camera lucida at a magnification of 3,500 diameters, obtained with a Spencer 1/12 homogeneous immersion objective and Spencer 15X compensating ocular with draw tube set at 150 mm., and drawing made at the level of the base of the microscope. The drawings were then enlarged by means of a copying camera lucida to 7,350 diameters. Having been reduced one-third in the reproduction they now appear at a magnification of 2,450 diameters.

W, the large sex-chromosome carrying female-tendency genes only.

w, the smaller sex-chromosome which also carries female-tendency genes only.

Z, sex-chromosome carrying a preponderance of male-tendency genes and also sex-linked genes.

38*Z*, same as *Z*.

38*w*, same as *w*.

37 to 1, autosomes.

EXPLANATION OF PLATE I

FIGS. 1–4. Cells from the amnion of males of the domestic turkey. The sex-chromosome is numbered 38*Z*. Autosomes from 37 to 33 are paired according to their size.

FIG. 1. Early prophase. The large chromosomes have not yet taken the characteristic peripheral position. Seventy-six chromosomes present.

FIG. 2. A soma cell aberrent in chromosomal number. This cell has the 12 large chromosomes of the first group, the 6 rod-shaped chromosomes of the second group, and 48 chromosomes of the third group. Ten of the smallest chromosomes of the third group are missing.

FIG. 3. Early metaphase showing gonomeric grouping of the largest chromosomes. Seventy-six chromosomes present.

FIG. 4. Early metaphase showing gonomeric grouping of the largest chromosomes, also some filamentous linkage between the members of the third group. Autosomes from 37 to 33 numbered. Seventy-six chromosomes present.

FIGS. 5 and 6. First spermatocytes from smear preparations. These cells are in the prophase stage. The *Z* chromosome bivalent in each cell is numbered 38*Z*, the autosomal bivalents from 37 to 1 according to their size. Thirty-eight chromosomes present.

PLATE I

Fig. 1

Fig. 2

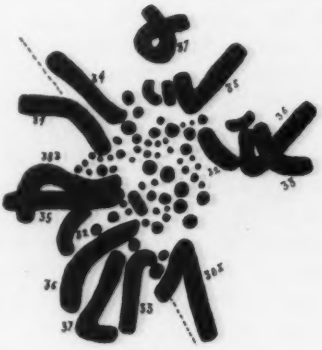

Fig. 3

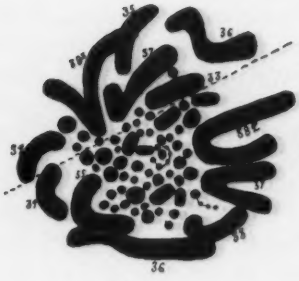

Fig. 4

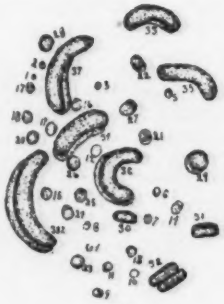

Fig. 5

Fig. 6

somes are often found in more or less modified forms at the beginning of mitosis.

The seventy-six chromosomes in the complex of the soma cells of the male (Figs. 1, 3, 4) appear to form a graduated series from the largest of the first group, 38*Z*, to the smallest of the third group. Of the twelve large chromosomes which form the first group numbers 38*Z*, 37 and 36 appear in most cases as J-shaped and 35, 34 and 33 as rod-shaped. The six chromosomes which form the second group are all of the rod type. They are in most cases sufficiently smaller than the smallest of the first group so as not to be easily confused with them, but the slight difference in the length of the individual members of the three pairs makes it sometimes difficult to distinguish one from the other. The third group, the globe-shaped chromosomes, range in size from those containing approximately as much chromatin as the smallest of the second group to very small ones. Many of these small chromosomes are so nearly of the same size that they can be compared with no degree of certainty. Figures 5 and 6 are first spermatocytes from smear preparations of testis material. The tetrad form of many of the chromosomes is plainly apparent. It would appear that gonial mates have the same spindle attachment and that they are of the same size. In these cells the haploid number is present and the grouping of the chromosomes and the size relations are the same as in the diploid number. The germ cells are so small that in sectioned material it is practically impossible to be sure of the small chromosomes; but in most cases the large chromosomes are easily made out as to number and form. However, the testis material lends itself well to smear preparations and when the cells are well pressed out, the chromosomes are sufficiently large and clear to distinguish their number and form.

EXPLANATION OF PLATE II

FIGS. 7 to 12. Cells from the amnion of females of the domestic turkey. *W* and 38*w* are the chromosomes that carry female-tendency factors only. Autosomes paired from 37 to 33.

FIG. 7. A prophase aberrent in chromosomal number. There are present the thirteen large chromosomes of the first group, the six rod-shaped chromosomes of the second group, and thirty-six of the chromosomes of the third group. Twenty-two of the smallest chromosomes of the third group are missing. Fifty-five chromosomes present.

FIG. 8. Prophase. Seventy-seven chromosomes present. Some filamentous linkage shown. Seventy-seven chromosomes present.

FIGS. 9 to 12. Cells in metaphase. The stippled line in each case shows a possible gonomeric grouping. This grouping in each case has been considered with especial reference to the twelve largest chromosomes. Filamentous linkage of chromosomes shown in each cell. Autosomes paired from 37 to 33. Seventy-seven chromosomes present.

PLATE II

Fig. 7

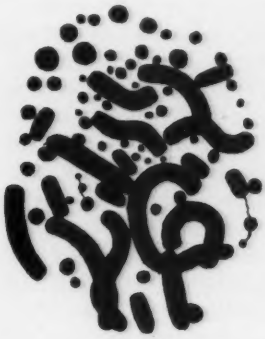

Fig. 8

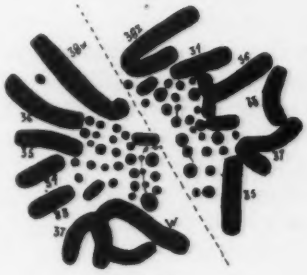

Fig. 9

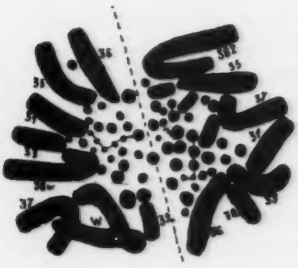

Fig. 10

Fig. 11

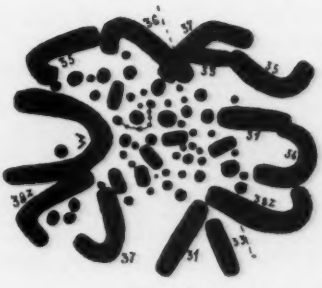

Fig. 12

As in the duck there is one more chromosome in the complex of the female than in the male. There are probably seventy-seven (Figs. 8–12). The odd chromosome is the largest in the complex. In these figures it is designated as *W*. This large chromosome, which is evidently a sex-chromosome, is quite pliant and apparently adjusts itself to other chromosomal regions and to the nuclear wall. For some time this chromosome was regarded as rod-shaped, but in anaphase it is J-shaped. However, it differs from the other J-shaped chromosomes in the complex in that the end that forms the loop of the *J* is tapering whereas the other J-shaped chromosomes are approximately uniform in diameter throughout their entire length. The next two largest chromosomes (38*w*–38*Z*) are apparently J-shaped. As in the duck it is difficult to say whether or not they are gonial mates. If the theory advanced in the former study concerning sex determination is correct then they are not gonial mates, but one of them must be regarded as a homologue of the 38's in the cells of the male and the other as a *w* chromosome. The remaining chromosomes in the complex of the female appear approximately the same as those of the same numbers in the cells of the male.

As in the duck gonomeric grouping is evident. In the metaphase of males (Figs. 3, 4) and in the metaphase of females (Figs. 9–12). As in the duck ". . . in the cells of the male the largest chromosomes are grouped six on one side of the forming equatorial plate and six on the other. . . . In the cells of the female there are six on one side of the plate and seven on the other. . . . In every case there is in the group of seven, one chromosome which is larger than the others which has the characteristic form of the largest odd chromosome in the cells of the female, large at one end and taper at the other." (Werner, Biological Bulletin, Vol. LII, No. 5, May, 1927).

I have not been able to determine whether the two halves of a nucleus are of exactly the same size. It would appear that in some cases there is some discrepancy in this respect. Neither have I been able to determine whether or not homologous chromosomes are of exactly the same size in any one stage of mitosis. In pairing the chromosomes I have selected as homologues those that are more nearly of the same shape and length. It is possible that there is a difference in the amount of chromatin material in some or all of the homologues. The chromosomes of the male may contain more than those of the female or vice versa. This is a difficult question but one that should be investigated.

As in the duck filamentous linkage occurs in the somatic cells. In most cases observed the linkage is between members of the third group, the globe-shaped chromosomes. (Figs. 1, 4, 8, 9, 10, 11, 12.) In

other cases it was between members of the second group and some one member of the third group. (Figs. 1, 11). The numbers of chromosomes thus attached in linear arrangement range from two to seven. The filaments are in most cases one in number, although there are sometimes two. (Fig. 1). They are in all cases oxyphylic in character and are somewhat roughened or crinkly.

Discussion

The similarities between the chromosomal complexes of the duck and of the turkey are at once apparent. In each form there is in the male an even number of chromosomes, while in the female there is an odd number of chromosomes and one more than is present in the cells of the male. The same condition is found in the chicken (now being investigated). As in the duck it seems probable that the largest pair of chromosomes in the male complex are the *Z* or the sex-chromosomes. Since the large *W* chromosome has been found in the female of the three forms of the aves, it seems impossible to regard it as a planosome, or supernumerary. It must, then, be regarded as an odd chromosome and if such, it is reasonable to suppose that it is a sex-chromosome. Since it is found only in the cells of the female, it is evident that it is concerned only with femaleness. It is equally evident that it does not carry sex-linked characters but that this must be the function of some other chromosome in the female complex. The scheme proposed in the former article for sex-linkage and sex-determination in the duck is entirely applicable in the case of the turkey and it seems unnecessary to repeat it in detail in this article. The generalities are that the female tendencies are carried by the *Ww* chromosomes, the male by the *Z* chromosomes. In both sexes the sex-linked tendencies are carried by the *Z* chromosomes. The autosomes are in a balanced condition between maleness and femaleness. It follows that a zygote receiving a genic complex equally balanced between maleness and femaleness, plus that which contains genes for maleness only (the *Z* chromosome of the male) would of necessity become a male. A zygote receiving a genic complex equally balanced between maleness and femaleness plus the *Z* chromosome, which contains genes for maleness only, and in addition the *Ww* chromosomes which carry genes for femaleness only, would become a female. It would, of course, follow that the F_1 and F_2 generations would inherit as is usual in such sex-linkage and as has been outlined in the previous article.

Summary

1. The chromosomes in the somatic cells of the turkey agree in number with the chromosomes in the somatic cells of the duck. These "appear to be 76 chromosomes for the male and 77 chromosomes for the female. There is present in the cells of the female a long unpaired chromosome which is not found in the cells of the male. There is reason to suppose that there are probably among the remaining six largest chromosomes two more unpaired chromosomes, one of which, the largest, is probably homologous to the largest pair (sex-linkage) of chromosomes in the male complex, while the other, it is thought may be some one of the five remaining long chromosomes." (Werner.)

2. As in the duck the 76 chromosomes appear to fall into three general groups. In the duck these groups consist of six pairs of large chromosomes, including three J-shaped and three rod-shaped; nine pairs of short rod-shaped chromosomes; and twenty-three pairs of globe-shaped chromosomes. In the turkey the first group consists of six pairs of large chromosomes, including four pairs of J-shape and two pairs of rod-shape. The second group consists of three pairs of short rod-shaped chromosomes; the third group consists of 29 pairs of globe-shaped chromosomes, which as in the duck, form a closely graduated series.

3. As in the duck there appear to be 38 bivalents in the primary spermatocytes of the male. These agree with the somatic cells in size gradations.

4. Gonomeric grouping occurs in the amnion cells as it does in the duck.

5. Filamentous linkage occurs in certain stages of the prophase and metaphase. This also agrees with the condition found in the duck.

6. The sex-mechanism appears to be of the *WwZ–ZZ* type similar to that found in the duck and in the moth *Phragmatobia*.

I desire to record my indebtedness to Dr. W. R. B. Robertson for the material for this work and for his criticism of the major part of the work; to the Bausch and Lomb Optical Company for the use of microscopic equipment during the year 1929; to Dr. Mary Rose Prosser, President of Cottey College, and to Mrs. Elizabeth Ott for their influence in securing from the Spencer Lens Company the proper equipment for the completion of the work.

THE SIGNIFICANCE OF HYDROGEN ION CONCENTRATION IN THE BIOLOGY OF EUGLENA GRACILIS KLEBS

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(From the Physiological Laboratory, Princeton University)

Introduction

Euglena gracilis Klebs is a common and important constituent of certain aquatic communities, but is easily cultured under laboratory conditions. Hence, it is especially suitable for investigations in the ecology of a single species, and the present study is a contribution to that field. As Allee (1930) pointed out in his presidential address before the American Society of Ecologists, we do not begin to know as much about the morphology and physiology of individual species as is desirable, and the mere cataloguing of organisms from different environments has yielded little of real value.

The present study is designed to show the effects of different H^+-ion concentrations in the external medium on *Euglena gracilis,* with other factors controlled in such a way that indirect effects, or effects from unknown variables, are reduced to a minimum. In nature, probably most of the effects of H^+-ions are indirect, but we can discover their true nature only by eliminating them under controlled conditions. For this reason, in the present studies, cultures free from all other organisms have been used.

Certain aspects of the physiology of *Euglena gracilis* are fairly well known, due to the researches of Klebs (1883), Zumstein (1899), Ternetz (1912) and others. In particular, its tolerance of high concentrations of citric and other acids was pointed out by Zumstein and Ternetz, both of whom made use of citric acid in the more or less complete elimination of bacteria from cultures of the *Euglena.* Kostir (1921) demonstrated that this high degree of tolerance for citric acid is, however, a species characteristic not generally true for the genus.

Zumstein emphasized the necessity of using bacteria-free cultures, asserting that the presence of bacteria materially depressed the division-rate. He believed that the increased division-rate in acid cultures was not a direct effect of the acid, but an indirect effect, through the elimination of the bacteria. His evidence was incomplete, but my own experiments demonstrate that his view was correct.

It is possible that other organisms may be similarly effective. Skadowsky (1926), studying cultures of mixed Protozoa in relation to H^+-ion concentration, arrived at an optimum pH for *Euglena gracilis* Klebs of about 3.8, a value very different from that which I have found in pure-line sterile cultures but not far from my finding in a series of cultures in which bacteria were present. Other Protozoa in Skadowsky's cultures showed different pH optima, his results indicating, for those species listed, almost no competition at optimum values. *Euglena gracilis* actually grows well in a wide pH range, as will be shown later, with a not very pronounced optimum. Therefore, may not this apparent (and very striking) optimum found by Skadowsky be due to the competition of one or more other forms at the true optimum for this species, depressing the division-rate of the *Euglena* below that at which it has no competition? Interspecies competition is very real, certainly applying to Protozoa as well as higher forms; and, in a case like that in question, it may mask the real responses of the individual species. The results obtained by Skadowsky may well apply, therefore, to *Euglena gracilis* in the community which he studied, but not to this species when isolated from others. The responses under the later conditions are fundamental to the particular species considered, but subject to modification by the presence of other forms. In studying the responses of an organism to an environmental factor under natural conditions, one must remember that the results of such findings apply only to the special complex community in which the study is carried out.

As far as the writer is aware, no observations of the present nature have previously been carried out on any of the Euglenoidina. A few related studies on the alga, *Chlorella,* have been made by Warburg (1919) and Wann and Hopkins (1927). A very good summary of studies on pH in relation to Protozoa, ciliates in particular, is that of Darby (1929), whose observations are extended in a later paper (1930). The earlier paper contains a good bibliography. A recent paper of general interest, which emphasizes the significance of CO_2 in influencing the pH of natural waters, is that of Powers (1930). A most satisfactory summary of the relations between pH and fresh-water and marine organisms is that by Bresslau (1926). A long bibliography is appended. Skadowsky's paper is of similar general interest.

Thanks for special favors in connection with the present study are due to Dr. W. B. Baker, of Emory University, for verification of my identification of the first specimens used; to Dr. C. H. Philpott, of Harris Teachers' College, for a demonstration of the technique of sterilizing *Paramecium;* and to the Digestive Ferments Company, of Detroit, Michigan, for information connected with the analysis of their

product, "Bacto-peptone." To Professor E. Newton Harvey, I am especially obligated for continuous advice and assistance in technical aspects of the problem, to say nothing of the inspiration gained from association with him in his laboratories.

Experimental Methods

The apparatus and methods of procedure used were, of course, necessitated by the desire to maintain unvarying from culture to culture all important factors, except pH, in the growth of *Euglena gracilis*. These factors are not only those affecting growth or reproduction, but also the limiting factors for photosynthesis. (Blackman, 1905; Stiles, 1925; Spoehr, 1926.)

The following physico-chemical conditions were subject to control: Temperature; frequency and intensity of light; a sufficient quantity and quality of food for both saprophytic and holophytic nutrition; in certain cases, carbon dioxide and oxygen tensions; hydrogen ion concentration.

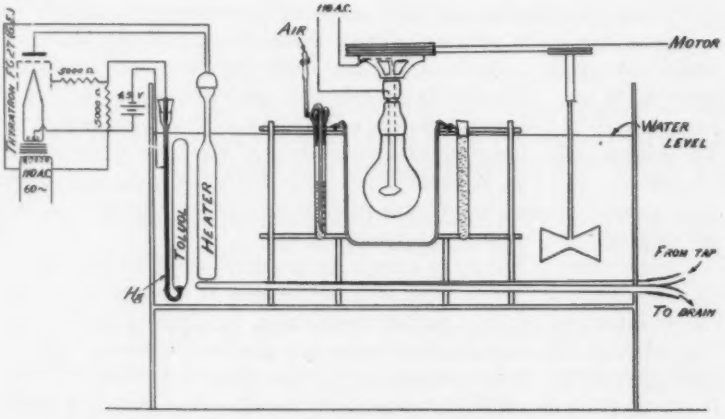

Fig. 1. Diagram of apparatus used in experiments.

Biological factors considered were: Absence of (1) a complicated life-cycle, (2) racial differences, and (3) other organisms; uniform density of organisms in all cultures at the beginning of an experiment; uniformity in organisms used for inoculation,—secured by using stock cultures of the same age and pH (in all but one experiment with etiolated *Euglena*).

For temperature control a water thermostat was adopted. This is illustrated in Fig. 1. The container is a metal tank, all inside parts

painted black. The temperature balance is maintained between a loop of copper pipe through which tap-water flows, and a knife heater. (A significant amount of heat is also derived from the illuminating source.) The regulation is by means of a Thyratron tube in circuit with a toluol-mercury thermo-regulator, as described by Loomis, Harvey and MacRae (1930). Temperature fluctuations, as determined with a Beckman thermometer, are less than 0.01° C.

As a source of light a 60-watt Mazda Daylight Lamp (frosted) was used. This was rotated about its vertical axis (at about 150 revolutions per minute) to provide uniform total distribution of radiation in all directions. However, since experiments with a 40-watt lamp resulted in rates of division as high as those with the 60-watt, the light intensity used was not limiting photosynthesis. As controls for the detection of photosynthesis and other light effects, identical cultures in absolute darkness were maintained in all experiments. The culture-tubes were covered with several coats of black varnish, and, further, separated from the source of light by an opaque metal screen.

In the tank the water was kept in constant circulation to maintain uniformity of temperature. The lamp was not suspended directly in the water, but inside a Pyrex cylinder closed below and open above (a beaker was used). Much of the heat from the lamp was conducted away in the air. Between this Pyrex wall and the cultures (in Pyrex test-tubes 18 × 150 mm.,) was a water thickness of 5.2 centimeters. The total distance from the axis of rotation of the lamp to the center of each test-tube was 12 centimeters. Distilled water was used in the tank, to avoid deposition of films of carbonate on the glassware. The test-tube rack is of aluminum, painted black.

Temperature is known to have a marked positive effect on rates of photosynthesis; but death from high temperature is accelerated in high concentrations of H^+-ions, as has been shown by Chalkley (1930) for *Paramecium*. In a preliminary experiment with *Euglena gracilis* I have observed the latter phenomenon at temperatures as low as 35° C. In the selection of a suitable temperature for the experiments one must, therefore, compromise. I have actually used temperatures of 29° and 29.5° C.

Bacto-peptone, "Difco" Standardized, was selected as the basic culture medium. Previous workers have used "peptone" chiefly, a fact which makes my observations the more comparable with theirs. Furthermore, this medium is not only sufficient for saprophytic nutrition, but also permits photosynthesis without the addition of other media, —at least between pH 8.5 and 3.5. In addition, Bacto-peptone is in itself a very well buffered system, and is therefore especially suitable

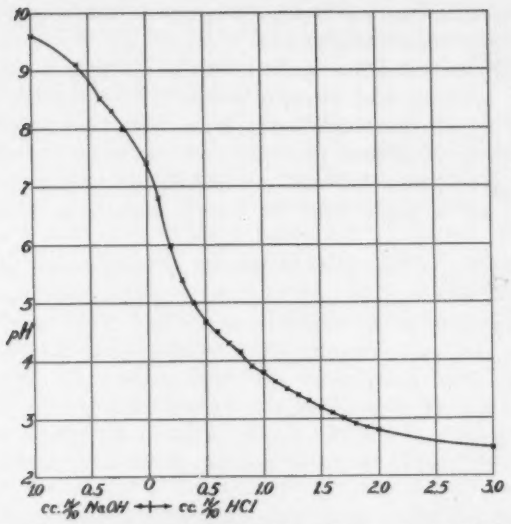

FIG. 2. Buffer curve for 1 per cent Bacto-Peptone in distilled water.

The original volume, to which were added the indicated quantities of NaOH and HCl, was 10 cc.

for studies of H^+-ion concentration (Fig. 2). Even within the pH range at which the buffer action is least, the pH changes produced by the growth in it of *Euglena* are, during the periods of experiments, not great (Table I).

The concentration of peptone used—dissolved in glass-distilled water—has been 1.0 per cent. With the density of organisms studied, no

TABLE I

Change in pH of cultures during an experiment lasting 48 hours.
These figures are from the experiment plotted in Fig. 5.

Initial pH	Final pH	
	In Light	In Darkness
8.10	8.13	8.07
7.65	7.72	7.70
6.71	6.87	6.87
5.90	6.11	6.15
4.60	4.64	4.84
3.56	3.55	3.55
2.96	2.81	2.89
2.52	2.39	2.48

greater growth is observed in this concentration than in 0.5 per cent, but its buffer value is somewhat greater.

The different pH values studied were produced by additions of hydrochloric acid, an acid probably completely dissociated, and which hardly, if at all, penetrates living cells. Its anion, furthermore, is such a common constituent of living matter as to be a relatively insignificant factor. The culture medium was always freshly prepared, and autoclaved but a single time for twenty minutes at fifteen pounds pressure.

This peptone is free from carbonates or bicarbonates in detectable quantity, and as a source of CO_2 for the organism it furnishes no more than that produced by the oxidation of the food-stuffs of which it consists. The medium is, therefore, free from a source of CO_2 that might be released with increased H^+-ion concentration. In any case, with peptone as the culture medium, the experimental results indicate that the major growth of *Euglena gracilis*—even in the light—is not due to photosynthesis, and is, therefore, not dependent on a supply of carbon dioxide.

The oxygen-tension, while apparently not a significant factor in the decomposition of CO_2,[1] is a very important factor in saprophytic nutrition. In cultures sealed from the air, the photosynthesis of the contained organisms provided the oxygen supply,—a supply which varied with the extent of photosynthesis. The greater the rate of photosynthesis, the greater the oxygen production, and, presumably, the greater the growth due to the oxidation of the organic foods in the medium. In aërated cultures, on the other hand, the organisms always had available a supply of oxygen (as well as carbon dioxide) in more or less complete equilibrium with the air. In cultures only initially aërated, and with access to the air at the surface, growth was somewhat limited by the oxygen-tension; but, in several experiments, a constant saturation with air was maintained.

The only factor intentionally varied from culture to culture has been the H^+-ion concentration. The range studied with most care has been between the limits of about pH 8.5 and 2.4, but the absolute limits of life have been approximately determined.

For measuring H^+-ion concentration, the quinhydrone electrode method was adopted. It is dependable to 0.02 pH, or less, below a pH of about 8.5, and is quite as convenient and rapid in use as the colorimetric method,—at least for pigmented solutions. By this method

[1] Harvey (1928) has shown that decomposition of CO_2 can take place in absence of oxygen. The method which he used with marine algæ, *i.e.*, luminous bacteria, as the indicator, was applied to *Euglena gracilis* with results similar to those obtained with the algæ.

it is possible to determine with accuracy such pH changes as occur in an active culture during the period of an experiment, the direction of change giving a clue to the occurrence or absence of photosynthesis. The values were recorded to 0.01 pH.

The stock cultures were grown in peptone medium unmodified by the addition of acid (pH between 7.2 and 7.9), with continuous artificial illumination, at room temperature. The chlorophyl content of the organisms did not vary materially from culture to culture. One study, however, was made with etiolated *Euglena* from a stock culture of a lower pH. The differences in division-rate between the green and etiolated ones were not great. Hence, slight differences in the chlorophyl content could not make appreciable differences in the results —except when using inorganic media alone.

Because of the so-called allelocatalytic effects of Robertson (1922), or a decreasing division-rate with increasing numbers (Jahn, 1929), it is necessary to compare cultures which begin with approximately the same numbers of organisms. By using for inoculation stock cultures that, to the eye, appear to be of about the same density, one can approach this condition. In any one experiment, each culture was inoculated with the same volume of stock culture. The variation in numbers per unit volume, as determined by actual counting in every case, was less than 10 per cent.

For counting, I have used a Rafter Counting Chamber, 1.0 cc. capacity, with a squared disc in the microscope ocular. Before counting, the organisms were killed and allowed to settle on the bottom. They were killed by heating to 65° C., a temperature at which they are coagulated but do not disintegrate under any of the observed conditions. The counts by this method were consistent to under 10 per cent (totals of ten counts each, from different parts of the chamber), even in cultures of lowest density of population. The error in the initial count probably determines the total error in counting. It is not greater than 10 per cent.

The chief source of error in the present experiments is in this determination of the numbers of individuals at the beginning and end of "runs." Slight differences in temperature, light, CO_2-tension, and other physico-chemical factors, would probably not produce variations greater than those between different counts of the same culture. The possibility of deviation from the true results is 10 per cent on the basis of counts alone, this probably determining the error in the experiments. We are not justified, therefore, in emphasizing any differences between cultures unless they are of a greater order of magnitude than about ten per cent—though consistent differences of less than that are probably real.

Genetic variables have been eliminated as far as possible by the use of organisms all descended from a single ancestor—a pure-line or clone. Variation due to complicated life-cycles has not been demonstrated in *Euglena*. (Reports of conjugation have been received with much scepticism.) Encystment occurs, but it was not observed in the course of any of my studies on pH. I found cysts in old stock cultures, of course, but none in the test-tube cultures after the two, three or four days of my experiments. Encystment is, therefore, not induced by any pH within the range 8.5–2.4. This is rather interesting, for encystment of some ciliates seems to be related to pH (Koffmann, 1924; Darby, 1929). I have found no evidence for a regular cycle involving encystment in *Euglena gracilis*, nor am I aware that any previous workers with this species have found such a cycle. Reproduction is, according to my observations, due solely to longitudinal fission. In the present study, the rate of reproduction is taken as the chief criterion of the effects of pH. This rate has been determined by considering the initial and final counts in a culture the first and last terms of a geometric progression with two as the common ratio.

The method used in establishing a *sterile*, pure-line culture was that used by Hargitt and Fray (1917) and Philpott (1928) for *Paramecium*. The parent individual for the present experiments was isolated October 2, 1930 and washed by transfer from one to another of five drops of sterile medium, being left in each for two or three minutes.[2] This individual and others sterilized at the same time were separately transferred to test-tubes, each containing about twenty cubic centimeters of sterile medium. Each culture began to appear greenish within about a week. Absence of other organisms was verified by making plate cultures on nutrient agar. The *Euglena* appeared on the agar, and reproduced rapidly, but no other forms were observed.

Experimental cultures were maintained sterile by ordinary methods of bacteriological procedure. Cultures sealed from the air were closed with sterilized, paraffined corks, tinfoil covers for corks having proved toxic in preliminary experiments. Air bubbled through sterile cultures was always sterilized by first passing it through sterile cotton, as illustrated in Fig. 1.

Results

Preliminary studies demonstrated that the previously reported increase in numbers of *Euglena gracilis* with increased concentrations of citric acid was largely related to the H^+-ion concentration. Maximum

[2] Parpart's criticism (1928) of the method of Hargitt and Fray does not apply in the case of *Euglena*, since it is extremely doubtful if *Euglena* ever ingests solid food.

growth in cultures made up with either hydrochloric, sulphuric, oxalic or citric acid occurred at pH 3 to 4, if no sterile precautions were observed. At higher pH values bacteria were numerous, especially near neutrality, but at the "optimum" few were present. Media adjusted to pH 3.6–3.7 by valeric or salicylic acids were fatal to *Euglena gracilis*, although the organisms thrived in more acid cultures prepared with the other four acids. The easily penetrating acids, as one should expect, proved fatal. The effect of the other acids was, however, obviously related to H^+-ion concentration, being independent of the kind of acid used. Subsequent experiments have shown that the increased growth at low pH is not a real effect of pH, but is an indirect result of the elimination of bacterial competition at these values.

In studies on a sterile pure-line, carried out in 1925, the maximum division-rate when CO_2 was the only acid added occurred at about the same pH of the medium as when hydrochloric acid is used,—*viz.*, pH 6.5 to 6.9. Concentrations of CO_2 above that represented by 5 per cent saturation at room temperature were not accompanied by increased division-rates under the conditions of the experiments. From these early observations I saw the desirability of, first of all, determining the relations between pH, as such, and the rate of reproduction of *Euglena gracilis*.

Sealed Cultures

Typical results from a series of sealed cultures are plotted in Fig. 3. Not much weight can be placed on the irregularities in the figures from the controls in darkness, but we can say that no striking effects are observable. The exhaustion of the limited oxygen supply, and the impossibility of its replenishment, make continued growth impossible at any pH in the dark.

An interesting collateral experiment was undertaken in this connection to determine whether or not this *Euglena* can live under anærobic or near-anærobic conditions. Similar cultures, in tubes with capillary necks, were exhausted as completely as possible with a vacuum pump. Each culture tube was then sealed at the neck in a flame. One set was placed in darkness, the other, in the light. Fairly rapid reproduction occurred in the latter. In the culture in darkness, however, the organisms were all motionless within twenty-four hours, and within another day were all encysted. They died in the encysted condition, for, when air was readmitted by breaking the sealed tip they did not excyst, nor did they excyst when transferred to fresh medium.

These results may be explained in the following way: In both cultures traces of oxygen and carbon dioxide remained after the exhaustion. In the light, a trace of the latter would be sufficient to initiate photo-

synthesis. This process once begun, the *Euglena* would be self-sufficient for both oxygen and carbon dioxide. But in darkness, after the little oxygen present had been used, the organisms were in complete absence of oxygen—physiologically—and could not replace it. Incidentally, it is of interest to note that this is the only means I have yet found to produce encystment in this form.

From this collateral experiment we are able to say that the low

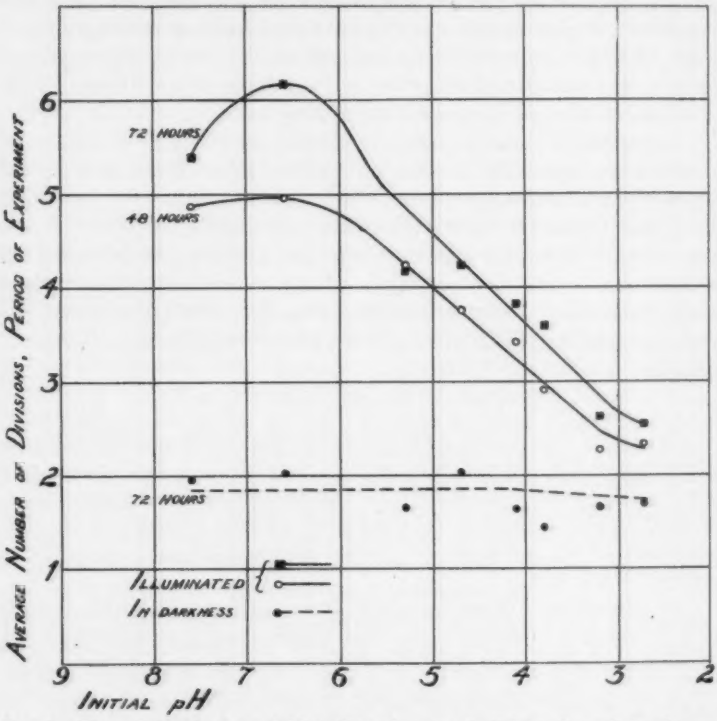

FIG. 3. The relation between initial pH and rates of division in sealed cultures.

division rates in darkness, as represented in Fig. 3, were probably due to the decreasing oxygen tension. It is doubtful if this effect is correlated with H^+-ion concentration.

In the light, on the other hand, a pronounced optimum at about pH 6.7 was apparent in all series. (This optimum has been, in another experiment, definitely established as below pH 6.9 and above pH 6.2.) The difference in rate between growth in light and in darkness must

be due either directly to the products of photosynthesis or to the increased oxygen supply available from the decomposition of CO_2. Since this difference is much less marked in aërated cultures, as will be shown later, it is obvious that most of the increased growth in the sealed cultures is only indirectly due to photosynthesis. It is actually due to the oxidation of foods in the medium, but is made possible by the oxygen produced during photosynthesis. *Euglena,* combining as it does two forms of nutrition, uses more oxygen in its normal metabolism than does a completely holophytic organism—the latter always producing, in good light, more oxygen than is required in its own metabolism. If this latter statement were not true, of course, life on earth would be impossible.

Reproduction is fairly rapid between pH 7.6 and 4.0, but there is a pronounced decrease in rate on both sides of the peak,—the rate at the peak being as great as in initially aërated cultures. Photosynthesis is, obviously, going on wherever growth in the light exceeds that in the dark, and this difference in rate is proportional to the amount of photosynthesis. Therefore, it is apparent that, under conditions stated, a definite optimum for photosynthesis exists in this form, and that it is near pH 6.7. Furthermore, the oxygen production at this pH is great enough to maintain reproduction at near the maximum rate observed in media in equilibrium with the air.

While making the counts at the end of the experiments just described, I observed in the most acid cultures a considerable number of organisms attached to each other in pairs. The point of attachment was the posterior tip, the last part to divide in longitudinal fission. I repeated this observation with a stock culture, of pH 2.9, heavily inoculated with *Euglena,* and maintained under constant illumination. This was first examined after forty-eight hours. At this time many of the organisms present were attached in pairs, as previously described, but, in addition, there were groups of three and four individuals composing these multiple monsters—and always all individuals were joined at the same point, the posterior tip. Table II gives their relative numbers in samples of uniform volume counted at the end of forty-eight hours after inoculation. Figure 4 consists of camera lucida drawings of several of these, and one or two other abnormalities which appeared with them. In every case in which the individuals were of approximately equal size, the connection was purely at the surface—no cytoplasmic connection being observable even with most careful examination under oil immersion.

The "colonies" of three or more individuals assumed the shape of rosettes, in appearance reminding one of small colonies of colonial algæ.

TABLE II

Relative Numbers of Single Individuals and Multiple Monsters in Ten Equal Volumes, from a Culture at pH 2.9 Examined 48 Hours after Inoculation

Single Individuals	Multiple Monsters		
	Double	Triple	Quadruple
24	11	1	
25	13		
16	12	1	
21	9		1
23	9		
19	8	4	
28	17	1	1
23	18	3	2
23	7	1	
23	14		
Average 22.5	11.8	1.1	0.4

Their progress through the medium was very irregular, depending apparently on the resultant of the vectors represented in the aggregate. Groups of as many as six individuals were observed, always attached at the point last to divide in their typical form of reproduction.

The effect is entirely on the surface, and is dependent on the H^+-ion concentration,—since it always occurs to a greater or lesser extent below a pH of about 3.5, and is equally common in illuminated or darkened, sealed or aërated cultures. It is apparently permanent. Subsequent

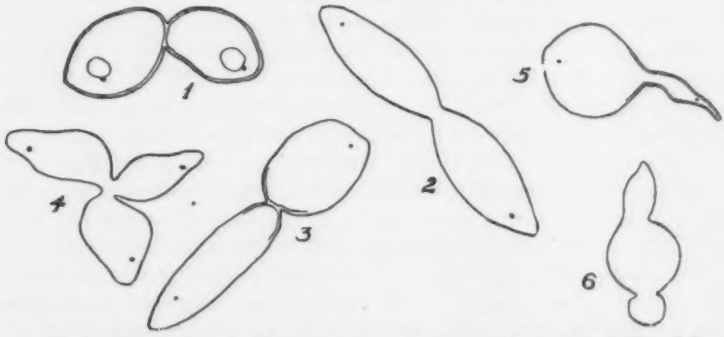

FIG. 4. Camera lucida drawings of monsters associated with media of low pH. 1–3, typical double monsters; 4, a triple monster; 5–6, other types of abnormalities observed. In 5, the cytoplasm was continuous between the "bud" and the parent organism.

examinations of the stock culture showed that most, if not all, of these multiple monsters never separated into individuals, but sank to the bottom and died in a few days. The cultures slowly developed, however, presumably habilitated by those individuals that had not been affected.

As the culture began to assume again the appearance of life (there was a period of a few days in which, to the eye, it seemed to have died out) an additional morphological effect of low pH began to be evident. The organisms now developing in the culture could be distinguished, but were not green. Examination under the microscope showed apparently complete etiolation, commonly extending in part to the stigma. The stigma was much reduced in size,—indistinguishable, in fact, in some individuals. Although several causes of etiolation have been found in *Euglena gracilis*, that due to low pH has not been previously reported.

Zumstein brought about etiolation by keeping cultures in an organic medium in complete darkness. I have repeated this, using sterile pure-line cultures. Zumstein also stated that the green form became colorless in "very rich organic medium." Ternetz described another hyaline form, differing from those observed by Zumstein in being permanently colorless. This form had completely lost the stigma, as well as chlorophyl. The type of etiolation associated with low pH is, however, not permanent. It may be similar to that caused by darkness or a "very rich organic medium," or may be identical with the latter, or with both. In any case, inoculation of culture medium at pH 7.2 with etiolated organisms from that at pH 2.9 yielded a normally green culture in a little more than a week.

At about this same time a stock culture was prepared to use in a study of the possible effect of initial adaptation in changing the shape of the pH—division-rate curve. This culture was adjusted to pH 4.6. As in the more acid cultures etiolation took place. When used for inoculation, the culture did not appear green to the eye, but when held to the light showed a rich growth of organisms. Under the microscope these individuals appeared colorless.

I have no explanation to offer as to the mechanism of this effect, but it is certainly associated with a high concentration of H^+-ions. Etiolation of *Euglena gracilis* ordinarily occurs in darkness at any pH, but, even in the light, and with continuous illumination, it occurs below pH 5 $\pm$. This etiolation, either in darkness or at low pH in the light, is evident on microscopical examination after only forty-eight hours.

Aërated Cultures

The next experiments were designed to provide a sufficient supply of oxygen in both light and darkness. Figures 5 to 7, inclusive, represent typical results,—the two former under conditions of initial aëration only, and Fig. 7, under conditions of continuous aëration as compared with initial aëration only, in identical cultures inoculated at the same time.

Air from a storage tank was bubbled through the cultures at a

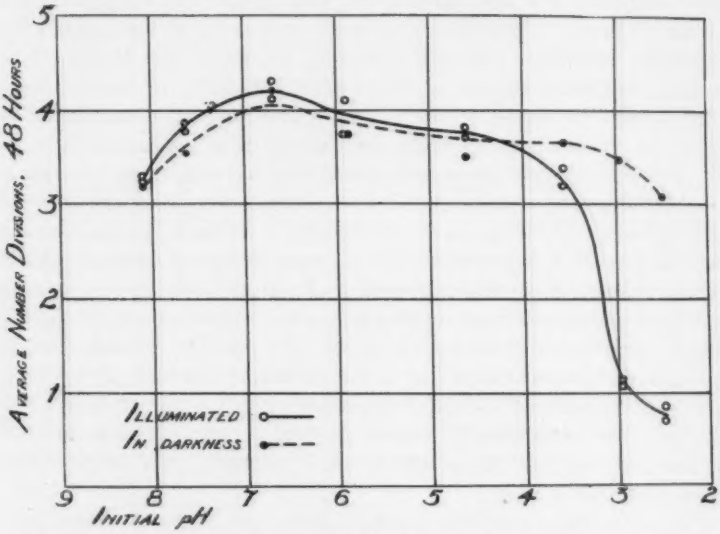

Fig. 5. The relation between initial pH and rates of division of normal green *Euglena*, in cultures initially aërated, and exposed to the air through cotton plugs.

vigorous rate, with shaking, for two minutes. The cultures were in flasks, each containing 80 cc. of medium and each adjusted to a particular pH. The aëration was carried out immediately after the introduction of organisms for inoculation. Following aëration, most of each culture was divided among three or four sterile test-tubes plugged with cotton, each containing 15 to 20 cc.,—two of these to be illuminated, and the other or others for a control in darkness. (During the experiments these cultures had access to the air through cotton plugs.) The pH was then determined in the medium remaining, and the final portion was used for determining the density of organisms. Each culture was shaken just before each transfer in order to maintain uniform distribu-

tion of the contained organisms. Agreement in counts from culture to culture was very close, and, since the initial volume of inoculating culture was the same in each, this indicates that the samples counted fairly represented the cultures used in the experiments.

The results from the initially aërated cultures are very different, both under illumination and in darkness, from those obtained from sealed cultures. In the experiment plotted in Fig. 5, in which normally green organisms were used, there was, above pH 4, a surprisingly small difference in division-rate between those in light and those in darkness. This, of course, is conclusive evidence that the major growth of this *Euglena* in the medium used is dependent on saprophytism, and not

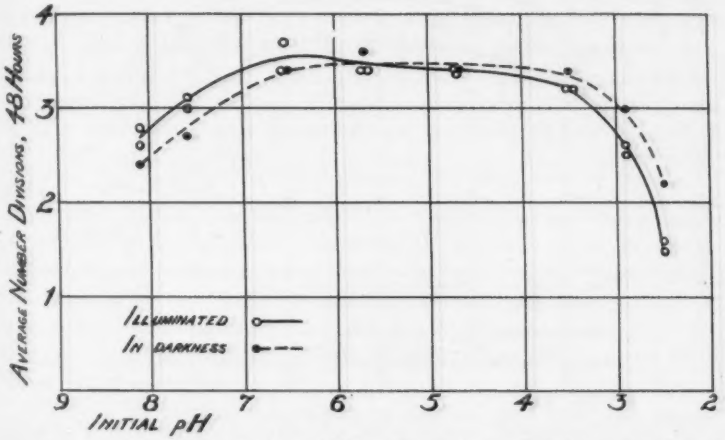

Fig. 6. The relation between initial pH and rate of division of etiolated *Euglena*, in cultures initially aërated, and exposed to the air through cotton plugs.

photosynthesis. However, there is a slightly greater growth in the light between pH 8.1 and 4.6. This is less than 10 per cent greater; but in another experiment I found a difference of 15 per cent in the same direction, at pH 7.5 and 6.6, under conditions otherwise the same. It is probably, therefore, a real difference, and indicates that photosynthesis plays some part in growth.

At about pH 4, on the other hand, the curve of growth in the light falls away rapidly, whereas that of cultures in the dark remains at a fairly high level. This indicates the presence of a photodynamic compound.

Comparison of Fig. 5 with Fig. 6 gives a clue to the nature of this compound. In Fig. 6, the results of experiments begun with etiolated

individuals are recorded. At the alkaline end of the range there is, again, evidence of photosynthesis. This is not unreasonable, for examination of the *Euglena* under the microscope showed the presence of small quantities of chlorophyl, even after only forty-eight hours in the cultures. On the other hand, there is no evidence of photosynthesis below pH 6. There is some evidence of a photodynamic effect toward the acid end of the range, but it is not nearly so pronounced as in the

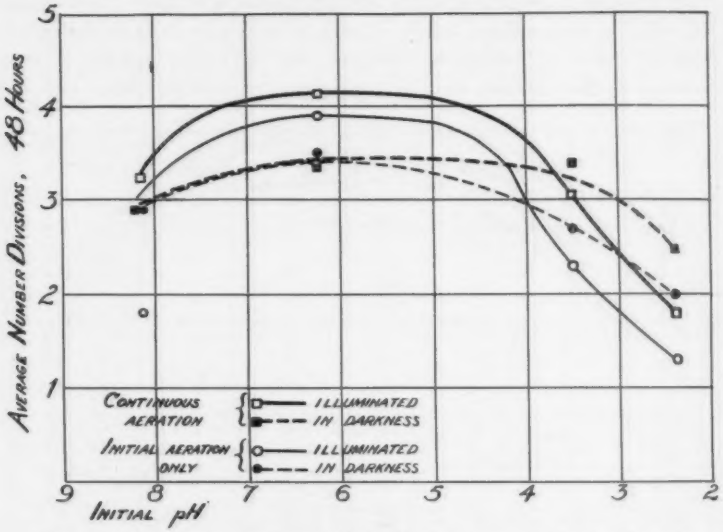

Fig. 7. The relation between initial pH and rates of division in cultures continuously aërated compared with that in cultures only initially aërated but subsequently exposed to the air through cotton plugs.

The flat top of the curves representative of continuously aërated cultures is based on another experiment, in which maximum rates were observed also between pH 7 and 8, and between pH 5 and 4. For convenience, only four pH values were selected in each "run." The very low rate of the initially aërated, illuminated culture at pH 8.1 + was due to bacterial contamination.

series begun with green organisms. This suggests that the photosensitizing material is either chlorophyl or one of its derivatives. Since etiolation occurs at about the same pH values, we cannot be certain that the chlorophyl itself is the material. In any case, it seems strange that an organism could be made photosensitive by one of its normal constituents. As far as I know, this is the first report of this effect. A photodynamic effect of chlorophyl (production of hemolysis of mammalian erythrocytes) has been demonstrated by Hausmann (1909), but

a comparison with the observation herein recorded seems rather far-fetched.

The stock culture used to inoculate the series plotted in Fig. 5 was at pH 7.9, that used in the experiment with colorless *Euglena,* at pH 4.7. In spite of this initial difference in pH, no very evident adaptive effect of the low pH in the stock culture is apparent. The curves are not strikingly different except at the extreme acid end of the range, and there the principle difference occurs only in the light.

As in sealed cultures, the undivided, multiple organisms appeared in the more acid conditions.[3] In one of these initially aërated cultures the group of six, mentioned previously, was observed. This group can have evolved as follows: The first individual divided partly, forming individuals a and b. Then each of these divided in the same incomplete fashion, forming a, a′, b and b′. Probably two of the four now attached divided, forming the six. There are alternative explanations, but this is the more probable one, since it means a maximum of three divisions each for four of the final six individuals, with two divisions each for the other two. The number of divisions is in agreement with the average for the culture.

In order to determine the validity of conclusions based on cultures aërated only at the beginning of experiments, several series of tests were carried out with cultures through which air was bubbled continuously. For convenience, only four H^+-ion concentrations were selected in each experiment. As controls for the continuously aërated cultures (in both light and darkness) duplicates were initially aërated and closed with cotton plugs.

Figure 7 represents the results of such a test. The continuous aëration does produce a somewhat greater rate of division, due to the large supply of carbon dioxide and oxygen, but the curves representing the rates under such conditions are similar in shape to those obtained from cultures only initially aërated. We may conclude from this that the effects of pH in cultures initially aërated and having access to the air are not qualitatively different from those in cultures continuously in equilibrium with the air. The results here are, however, based on experiments lasting for only forty-eight hours. Over longer periods of time, the changes in standing cultures might tend to make the pH-division-rate curve approach the form of that found for sealed cultures.

Observations on aërated cultures furnish little or no evidence for a pronounced optimum for the rate of division. This rate is fairly uni-

[3] All multiple organisms were counted as if all divisions begun had been completed. Inasmuch as these divisions are probably never completed, but followed by death, this method eliminates part of the true depression of division-rate at low pH.

form over an extremely wide range of H^+-ion concentrations. A slight increase between pH 6 and 7 suggests an optimum there, but this is not nearly so apparent as that associated with photosynthesis alone.

All types of experiments consistently give us three explanations for the depression of division rate at low pH, exclusive of that associated with fatal injury to the organisms. (1) Etiolation, (2) production of multiple monsters which subsequently die, and (3) a photodynamic effect, all probably contribute to this depression. In addition, both high and low pH values are fatal—the limits found being pH 2.3 and 11.0 +. (*Euglena gracilis* will grow at pH 9.6, but its average rate of division is less than once in three days. No evidence of special effects at high pH has been obtained.)

It may be thought that the form of movement commonly observed in *Euglena,* and called variously "euglenoid movement" or "*Metabolie,*" may be induced by particular concentrations of hydrogen ions. In 1925, while working with *Euglena gracilis,* I found that organisms transferred from an acid medium to one distinctly more alkaline, or *vice versa,* began to go through this type of rhythmical contraction, and did not return to the normal swimming form for some time. The response is quite evidently associated with a change of environment, rather than with the nature of the new environment. It seems to the writer that the response to pH indicates that "euglenoid movement" is a modified avoiding reaction. It is certainly not a form of locomotion, for as a means of progress from place to place it is little, if at all, more efficient than Brownian movement.

Summary

1. The alleged tolerance of high concentrations of citric acid by *Euglena gracilis* Klebs is associated with H^+-ion concentration. This species of *Euglena* tolerates H^+-ion concentrations as high (or higher) when produced by hydrochloric, sulphuric or oxalic acids.

2. Even less acid solutions of valeric and salicylic acids cause death, however. This effect may be connected with the greater rates of penetration of these acids into living cells.

3. Increased growth of *Euglena gracilis* at high acidities is due to the elimination of bacterial or other competition at such pH values. It does not occur in a series of sterile cultures.

4. A sterile, pure-line, well-aërated culture of *Euglena gracilis,* in Bacto-peptone medium, showed little difference in division-rate between pH 7.7 and 4.5 in the light, and between pH 7.7 and 3.0 in darkness. An only slightly greater rate of division at about pH 6.7 suggests that a true optimum may exist at that point.

The absolute limits of life proved to be approximately pH 2.3 and 11.0 +.

5. There is a marked optimum pH for photosynthesis in this *Euglena*. Sealed cultures in the light invariably developed maximum growth at pH 6.5 to 6.8. Comparison with aërated cultures in darkness shows that the difference between growth of sealed cultures in light and in darkness is actually not due to the products of photosynthesis in the former, but to the use of the oxygen produced during photosynthesis in the oxidation of organic foods in the medium.

6. Quantitatively, enough oxygen is produced at pH 6.7, in photosynthesis by this *Euglena* to maintain its own reproduction at maximum or nearly maximum rate. *Euglena* may, therefore, be a significant element of the carbon cycle where it occurs in nature.

Under conditions of nearly complete anaërobosis in darkness it does not grow, but encysts, and subsequently dies.

7. In cultures of about pH 3.5 or lower, many individuals fail to complete normal division, remaining attached together by the surface at the posterior tip (the last part to divide in euglenoid fission). Two, three, four and even as many as six individuals have been found attached together, the higher numbers of individuals forming rosettes. This condition depends on pH, as such, occurring equally in light and darkness, in sealed and aërated cultures. Furthermore, it is apparently only a surface effect, as, although occasionally other types of abnormalities appear, in the type here described there is no connection between the cytoplasm of one individual and that of another.

8. More or less complete etiolation occurs, even under continuous illumination, below pH 5 ±. This is not permanent, the chlorophyl reappearing after the organisms are returned to a medium of lower H^+-ion concentration.

9. In cultures of low pH (below about pH 3.5) a photodynamic effect is present. This is far more marked with green than with etiolated individuals, which suggests that the contained chlorophyl, or a derivative of it, is the photodynamic compound involved.

10. Encystment does not occur as an effect of pH anywhere in the range studied, pH 8.5 to 2.4.

11. The so-called "euglenoid movement" is not induced by any particular pH, but may be brought about by transferring the organisms from the culture in which they have been living to one in which the pH is markedly different. This type of movement should not be considered a form of locomotion, but, rather, a modified avoiding reaction.

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THE RESPIRATORY FUNCTION OF THE BLOOD OF URECHIS CAUPO

ALFRED C. REDFIELD AND MARCEL FLORKIN [1]

(From the Hopkins Marine Station, Pacific Grove, California)

Urechis caupo is an echiuroid worm inhabiting sandy mud flats in the estuaries of the coast of California. Its characters and habits have been described by Fisher and MacGinitie (1928). Because of its large size, the simple nature of its circulatory and respiratory systems, and the fact that its cœlomic fluid is voluminous and contains abundant red blood corpuscles, it affords unusually suitable material for the study of respiratory problems. The present paper contains a description of those properties of the blood of *Urechis* which are of importance in respiration, together with certain observations designed to evaluate the significance of these properties.

The material used for these studies was collected in the Elkhorn Slough, a tributary of Monterey Bay. The authors wish to express their indebtedness to Dr. MacGinitie for assistance in procuring the animals and to Professor Fisher for the many courtesies received while they were at the Hopkins Marine Station.

I. The Blood of Urechis

Fisher and MacGinitie (1928) state that the cœlom is filled with bright red blood, the pigment being lodged in subcircular cells, about 0.025 mm. in diameter, which readily distort when crowded. There are also very numerous amœboid cells which when aggregated are yellow in color. We have found the color of the blood to vary, being frequently of a dull brownish-red color; less often, and particularly in smaller specimens, of a bright scarlet resembling the blood of vertebrates. A volume of 15 or 20 cc. may be secured from a single specimen.

The Blood

The plasma does not clot, and when separated from the cells is a pale yellow color; not infrequently it may be tinged with the corpuscular pigment. Under microscopic examination the cytoplasm of the corpuscles appears yellow and is seen to be filled with small, highly refractive granules. In addition, there are many granules of a brown pigment

[1] Fellow of the C. R. B. Educational Foundation.

in the corpuscles of some specimens. The occurrence of this pigment is variable and it may be nearly lacking in those specimens whose blood appears scarlet rather than brownish-red. In the center of the corpuscle is a clearer area, which, on staining, proves to be a small nucleus. The cœlomic cells of *Urechis chelensis* described by Seitz (1907) are apparently similar, being nucleated and containing yellow pigment granules (in preserved material).

TABLE I

Spectrometric Data of Urechis Hemoglobin

Wave Length	Oxyhemoglobin	Reduced Hemoglobin	Wave Length	Oxyhemoglobin	Reduced Hemoglobin
mμ	*E*	*E*	mμ	*E*	*E*
450.4	0.394	0.495	566.6	0.221	0.299
460.5	0.280	0.225	567.6	0.223	
470.6	0.226	0.169	569.7	0.245	0.273
480.7	0.189	0.160	571.7	0.285	
490.8	0.179	0.164	573.7	0.297	
500.9	0.174	0.176	575.7	0.309	
511.0	0.172	0.197	576.7	—	0.238
516.1	0.180		577.8	0.310	
521.1	0.192	0.218	578.9	0.297	
523.2	0.206		581.8	0.267	0.204
525.2	0.215		583.8	0.214	
527.2	0.231		585.8	0.168	
529.3	0.244		586.9	—	0.171
531.3	0.264	0.245	587.9	0.131	
533.3	0.276		589.9	0.102	
535.3	0.291		591.9	0.082	0.145
537.3	0.299		597.0	0.055	
539.3	0.306		602.0	0.042	0.096
541.4	0.312	0.277	612.1	0.032	0.073
543.4	0.310		622.2	0.025	0.064
545.4	0.292		632.4	0.022	0.059
546.4	—	0.298	642.5	0.024	0.056
547.4	0.276		652.6	0.009	0.049
549.5	0.255		662.7	0.016	0.046
551.5	0.243	0.313	672.8	0.004	0.038
555.5	0.214		682.9	0.008	0.036
556.5	—	0.313	693.0	0.003	0.028
559.6	0.197				
561.6	0.197	0.312			
563.6	0.202				
565.6	0.211				

The corpuscles appear to be surrounded by a strong membrane. On dilution of blood with distilled water, the cells swell but do not burst. Upon applying pressure to the coverslip when in this condition the membrane ruptures and the contents may be seen to flow out through a localized opening. The granules in the swollen corpuscles are in active Brownian movement, suggesting a fluid state of the interior of the cell.

In a one per cent saponin solution the cells swell, the granules remaining confined to the previous volume of the cells and appearing surrounded by a clear region. After a few minutes the membrane spontaneously ruptures and the granules flow out through the localized opening.

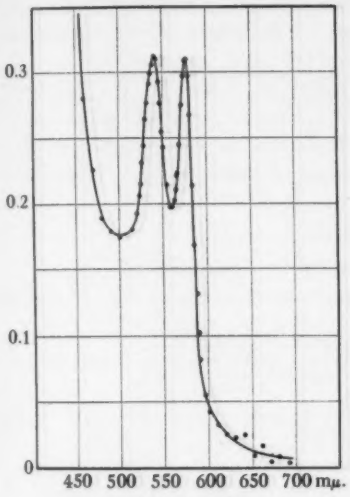

FIG. 1

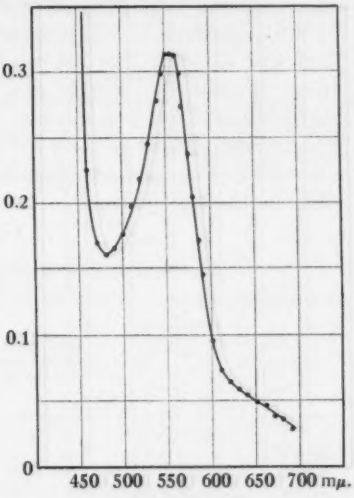

FIG. 2

FIG. 1. Absorption spectrum of oxygenated hemoglobin of *Urechis caupo*. Ordinates: extinction coefficient of solution of unknown concentration.

FIG. 2. Absorption spectrum of reduced hemoglobin of *Urechis caupo*. Ordinates: extinction coefficient of solution of same concentration as that shown in Fig. 1.

The Respiratory Pigment

That the corpuscles of *Urechis* contain hemoglobin is indicated by the spectroscopic examination of laked blood. Typical hemoglobin crystals may be obtained by allowing laked corpuscles to dry under a coverglass. For spectrophotometric examination a solution of hemoglobin was prepared by laking one cc. of corpuscles separated by centrifugation with 11 cc. of distilled water containing three drops of ether. To this solution was added 8 cc. of 4M ammonium sulfate brought to ca. pH 8 by the addition of ammonia. From this solution the corpuscular debris was filtered off and one volume of the filtrate diluted with four volumes of water. Filtration was repeated. The filtrate so obtained contained the original corpuscular content of hemoglobin diluted 1 : 100 and in the presence of 0.8 molar ammonium sulfate at a pH of approximately 8.

One sample of this solution was employed for measuring the absorption spectrum of oxyhemoglobin; another specimen was reduced by equilibration with hydrogen and used to obtain the spectrum of reduced hemoglobin. The solutions were perfectly clear. The measurements were made with a König-Martens spectrophotometer within five hours of the completion of the preparations. The length of the column of fluid was 3.3 cm. The extinction coefficients of these solutions, estimated for one cm. length, are recorded in Table I and illustrated graphically in Figs. 1 and 2. In Table II are recorded the wave length of maximum density in the α and β bands and the wave length of minimum absorption between these bands as obtained by various workers with various hemoglobins.

TABLE II

Spectrometric Characteristics of Various Oxyhemoglobins

Species	Wave Length of Maximum Absorption		Wave Length of Minimal Absorption between α and β Bands	Ratio of Extinction Coefficients at Maximum β and Minimum between α and β	Observer
	α Band	β Band			
	mμ	mμ	mμ		
Dog and horse....	575.6	540.4	558.1	>1.60	Hári (1917)
Dog.....	575–577	539–542	560.*	1.63	Kennedy (1926–27)
Horse.....	578.2	540.4	562.5*	1.58*	Vlès (1923)
Arenicola..	576.0	540.0	560.*	1.53*	Vlès (1923)
Marphysa .	578.0	540.0			Vlès (1923)
Cucumaria	579.	542.7	558.		Van der Lingen and Hogben (1928)
Urechis....	577.	542.	561.	1.58	

* Estimated from published data of observer.

These values for *Urechis* hemoglobin agree more closely on the whole with those found for the hemoglobin of the worm *Marphysa* and the holothurian *Cucumaria* and with Vlès' measurements of horse hemoglobin than with this author's data for *Arenicola*. The values for horse hemoglobin obtained by Vlès, from which he concluded that *Arenicola* hemoglobin differed from horse hemoglobin, do not agree with the values obtained by Hári and Foster for the mammalian pigment, which are very similar to Vlès' values for *Arenicola*. The shape of the absorption curve for *Urechis* does not agree exactly with the data recorded for mammalian oxyhemoglobin, particularly in the region about 510 mμ. Discrepancies in the shape of the curves may be attributed to the

presence of methemoglobin in the solutions, as Hári has pointed out; a fact which makes the direct comparison of the curves difficult. The data leave no doubt that the pigment of the *Urechis* blood is a hemoglobin, but the spectrometric evidence regarding the specificity of the hemoglobin cannot safely be interpreted.

TABLE III

Cell Volume and Oxygen Capacity of Urechis Blood

Specimen	Volume Red Cells	Oxygen Content	Oxygen Combined	Oxygen Combined per 100 cc. Cells
	per cent	*volumes per cent*	*volumes per cent*	*cc.*
3	36.6	—	—	—
4	26.4	—	—	—
7	40.3	6.30	5.80	14.4
		7.22	6.72	16.7
8	37.6	5.83	5.33	14.2
		5.70	5.20	13.8
		5.77	5.22	13.9
9	18.3	2.85	2.35	12.8
		2.87	2.37	12.9
10	35.3	5.72	5.22	14.8
		5.53	5.03	14.2
12	23.8	4.64	4.14	17.4
		4.36	3.86	16.2
		4.24	3.74	15.7
13	19.5	3.89	3.39	17.4
		3.70	3.20	16.4
14	23.2	2.90	2.40	10.3
		2.83	2.33	10.0
		2.66	2.16	9.3
15	28.6	4.53	4.03	14.1
		4.30	3.80	13.3
16	32.0	4.54	4.04	12.6
		4.78	4.28	13.4
21	—	3.70	3.20	—
20	—	4.43	3.93	—
		4.50	4.00	—
23	—	4.09	3.59	—
	—	4.05	3.55	—

Hemoglobin appears to occur in the musculature of *Urechis,* particularly in that of the foregut, or crop. In this structure, which is in a thin muscular tube of a bright pink color, the spectrum of oxyhemoglobin can be beautifully demonstrated with the microspectroscope. If the preparation is covered with a coverglass, the spectrum soon changes to that of reduced hemoglobin, except near the edges, where the oxyhemoglobin bands persist. Because of the absence of capillaries in this preparation it should form very advantageous material for the study of the function and properties of muscle hemoglobin.

The Quantity of Corpuscles and Hemoglobin in the Blood

The red corpuscles occupy from 18 to 40 per cent of the total volume of the blood when separated with the hematocrit (Table III). A gray layer of rather variable volume containing sperm or eggs and other cells separates between the red cells and the plasma. The oxygen content of the blood equilibrated with air was determined with the Van Slyke constant volume blood gas apparatus, using one cc. samples, and varies between two and six volumes per cent. Special care was taken to stir the blood before sampling because of the rapid rate at which the large corpuscles settle out. A one per cent saponin solution was used as laking reagent. These values are recorded in Table III and may be compared with the values found for other worms and other invertebrates containing hemoglobin in Table IV.

TABLE IV

Oxygen Content of Blood of Worms and Other Invertebrates (equilibrated with air)

Species	Oxygen Content	Pigment	Occurrence	Observer
Urechis caupo.....	2.66–7.22	Hemoglobin	in corpuscles	
Glycera siphonostoma	2.58–3.03	Hemoglobin	in corpuscles	Winterstein (1909)
Arenicola sp.....	5.70–8.70	Hemoglobin	in solution	Fox (1926) after Barcroft and Barcroft (1924)
Cardita sulcata.....	1–2	Hemoglobin	in solution	Winterstein (1909)
Pectunculus violaceus	1–2	Hemoglobin	in solution	Winterstein (1909)
Spirographis.....	8.10–10.0	Chlorocruorin	in solution	Fox (1926)
Siphunculus nudus...	ca. 2	Hemerythrin	in corpuscles	Winterstein (1909)

It is commonly believed that the inclusion of the respiratory pigments within corpuscles has made possible the superior oxygen capacity of the blood of vertebrates. This possibility does not appear to have been realized in the invertebrate stage of development, for *Arenicola* and

Spirographis, which carry their respiratory pigments in solution, have a greater oxygen content than *Urechis* and the other invertebrate forms in which oxygen is transported in blood corpuscles.

The concentration of hemoglobin in the corpuscles of *Urechis* appears to be much less than is the case in vertebrates. In Table III is recorded the estimated oxygen combined per 100 cc. of red corpuscles —allowance being made for 0.50 volumes per cent of oxygen assumed to be present in solution. The oxygen-combining power of the cells varies from about ten to seventeen volumes per cent. Drastich (1928) finds the following values for the hemoglobin content of the cells of vertebrates:—various mammals 29.5 to 34; various birds 29.54; *Rana esculenta* 24.85; carp 26.02 grams per 100 cc. corpuscles. Assuming the *Urechis* hemoglobin to have the same oxygen-combining power per unit weight as mammalian hemoglobin (one volume per cent oxygen capacity corresponding to 0.746 grams of hemoglobin per 100 cc.), *Urechis* corpuscles are estimated to contain 7.5 to 12.7 grams of hemoglobin per 100 cc. of cells. The *Urechis* corpuscle is then about one-third as effective in transporting oxygen as those of the vertebrates. It is to this fact rather than to a deficiency in the number of corpuscles that the low oxygen capacities of the blood are principally due.

TABLE V

Data on Equilibrium of Oxygen with Urechis Blood

Carbon Dioxide Pressure	Oxygen Pressure	Oxygen Content	Oxygen Dissolved	Oxygen Combined	Saturation
mm. Hg	*mm. Hg*	*volume per cent*	*volume per cent*	*volume per cent*	*per cent*
8.64	5.98	0.63	0.192	0.44	19.1
9.78	8.50	0.87	0.027	0.84	36.6
8.61	12.15	1.15	0.039	1.11	48.3
7.90	16.35	1.46	0.053	1.41	61.3
7.54	23.08	1.88	0.074	1.81	78.8
10.87	41.85	2.09	0.135	1.95	84.8
6.54	48.15	2.15	0.155	1.99	86.6
6.11	72.61	2.32	0.234	2.09	90.9
7.56	87.21	2.36	0.281	2.08	90.4
air	air	2.90	0.50	2.40	
		2.83	0.50	2.33	
		2.66	0.50	2.16	
				—	
				av. 2.30	100

Iron Content of Blood

Attempts to estimate the iron content of the blood by the method of Hall and Gray (1929) yielded rather discordant results. The values obtained were always of the order expected from the oxygen capacity of the samples.

The Equilibrium of Oxygen with the Blood

The oxygen dissociation curve of the whole blood has been determined using the Van Slyke constant volume apparatus for blood gas estimations and the Haldane analyzer for measuring the composition of the gas with which the blood has been equilibrated. Equilibration was carried out upon 3 cc. of blood enclosed in 250 cc. tonometers rotated for 20 minutes in a water bath at 19° C. Analyses were made immediately after equilibration in fear that the metabolism of the cells might alter the gaseous content were the samples allowed to stand. The carbon dioxide pressure was maintained approximating that obtaining in the blood *in vivo;* about seven millimeters. The data are recorded in Table V.

In estimating the combined oxygen from the oxygen capacity it is assumed that blood in equilibrium with air dissolves 0.5 volumes per cent oxygen, the solubility at lower oxygen pressures being proportional in accordance with Henry's law. The oxygen dissociation curve is plotted in Fig. 3.

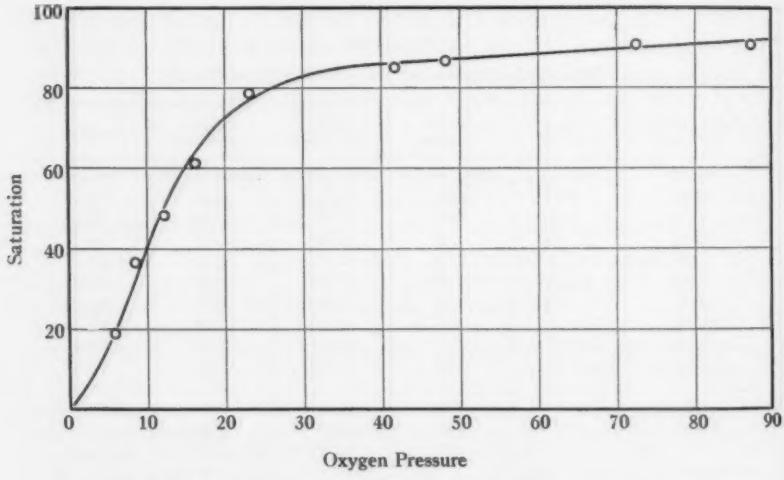

FIG. 3. Oxygen dissociation curve of blood of *Urechis caupo.* Temperature 19° C. For data see Table V. Ordinates: percentage of saturation; abscissæ: partial pressure of oxygen in mm. Hg.

The Effect of Carbon Dioxide upon the Oxygen Dissociation Curve

Samples of blood have been equilibrated with oxygen in the presence of carbon dioxide at pressures varying from 0.54 to 92 mm. Hg. The temperature of equilibration was 19° C. The results are recorded in Table VI. From this data, p_{50}, the oxygen pressure at which the blood would have been half saturated with oxygen has been calculated, assuming the curves to have the same shape as that drawn in Fig. 3.

TABLE VI

Data on Equilibrium of Blood with Oxygen at Various CO_2 Pressures

Specimens	Carbon Dioxide Pressure	Oxygen Pressure	Oxygen Content	Oxygen Dissolved	Oxygen Combined	Saturation	p_{50}
	mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*mm. Hg*
Urechis No. 15	0.54	10.40	1.96	0.03	1.93	49	10.6
	0.94	14.89	2.62	0.04	2.58	66	10.7
	8.60	7.10	2.16	0.02	2.14	55	6.3
	8.82	14.12	2.60	0.04	2.56	66	10.1
	19.60	8.40	2.25	0.12	2.23	57	7.2
	19.80	16.25	2.68	0.05	2.63	67	11.3
	29.40	12.55	2.20	0.04	2.16	55	11.2
	air	air	4.53	0.50	4.03	103.3	—
			4.30	0.50	3.80	97.4	—
Urechis No. 16	0.62	8.38	1.74	0.03	1.71	41.	9.9
	0.71	10.80	1.95	0.03	1.92	46.	11.7
	0.76	9.56	1.96	0.03	1.93	47.	10.1
	1.26	9.44	1.77	0.03	1.74	42.	11.1
	77.0	9.08	2.14	0.03	2.11	51.	9.0
	92.0	11.30	2.34	0.04	2.30	55.	10.1
	air	air	4.54	0.50	4.04	97.	—
			4.78	0.50	4.28	103.	—

These values, recorded in the last column of Table VI, make it appear that the affinity of the blood for oxygen is not influenced to a detectable degree by the pressure of carbon dioxide within the ranges of pressure examined. In this regard the blood of *Urechis* differs from that of most vertebrates and from that of *Arenicola*. In the latter form Barcroft and Barcroft (1924) found the typical effect of hydrogen ion concentration upon the oxygen dissociation curve. Recently Dill and Edwards (1931) have observed that in the blood of the elasmobranch, *Raia oscillata*, the effect of carbon dioxide upon the oxygen dissociation curve is absent or nearly so.

The Effect of Temperature upon the Oxygen Dissociation Curve

Oxygen dissociation curve data obtained from the same specimen of blood have been secured at two temperatures, 22° C. and 34.5° C. (Fig. 4). The carbon dioxide tension was about 12 mm. in both cases.

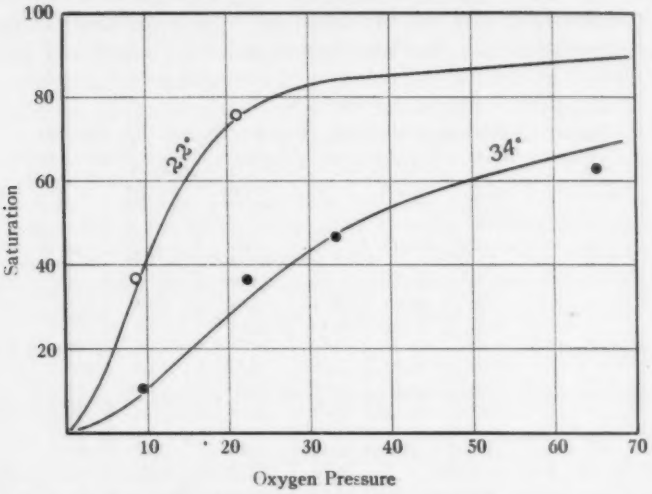

FIG. 4. Oxygen dissociation curves of blood of *Urechis caupo*, equilibrated at temperatures of 22° C. and 34° C. Ordinates: percentage of saturation; abscissæ: partial pressure of oxygen in mm. Hg.

The curve drawn through the data obtained at 22° C. is identical with that in Fig. 2 obtained from another sample of blood at 19° C. At 34° C. the points lie well to the right. The data are insufficient to warrant any conclusion with regard to the shape of the curve at the higher temperature, but it is clear that the temperature effect upon the oxygen equilibrium is large and of the same direction and order observed in vertebrate hemoglobin (Brown and Hill, 1923; Macela and Seliškar, 1925).

The Equilibrium of Carbon Dioxide with the Blood

Table VII presents the data obtained by equilibrating *Urechis* blood against various mixtures of carbon dioxide in air at 18.5° C. The analyses were made with the Van Slyke apparatus and the Haldane analyser. The oxygen capacity of the blood employed corrected for dissolved oxygen was 3.9 volumes per cent.

In order to facilitate comparison of the *Urechis* blood with that of

TABLE VII

Data on Equilibrium of Carbon Dioxide with Urechis Blood

Carbon Dioxide Pressure	Carbon Dioxide Content	Carbon Dioxide Dissolved (H_2CO_3)	Carbon Dioxide Combined ($BHCO_3$)	$\log \frac{(BHCO_3)}{(H_2CO_3)}$
mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	
0.9	3.32	0.09	3.23	1.597
3.3	6.13	0.35	5.78	1.217
7.22	8.90	0.76	8.14	1.029
12.40	11.00	1.30	9.70	0.873
22.0	14.15	2.31	11.84	0.710
47.2	19.15	4.96	14.19	0.456

other animals, and for the comparison of various experiments with this species it is convenient to relate the data to the logarithm of the ratio of combined ($BHCO_3$) to free (H_2CO_3) carbonic acid. This function changes approximately in proportion to the hydrogen ion concentration, which may be obtained by adding the appropriate pK value. Moreover, the total buffer value of the blood is also dependant upon this function. The quantity of carbon dioxide dissolved in the blood or present as H_2CO_3 (free carbonic acid) has been estimated assuming α, the number of cubic centimeters of CO_2 dissolved in one cubic centimeter at a pressure of 760 mm. Hg, to be 0.80. This value is slightly less than the value 0.827 given by Bohr (1897) for two per cent NaCl at 18° C.

The concentration of combined carbonic acid ($BHCO_3$) is obtained by subtracting the free carbonic acid (H_2CO_3) from the total carbonic acid. The estimated values of these quantities are included in the table.

The total buffer value of blood, β, is defined by the equation

$$\beta = \frac{-\Delta (BHCO_3)}{\Delta \log \frac{(BHCO_3)}{(H_2CO_3)}}$$

In Fig. 5 the values of ($BHCO_3$) are plotted against $\log \frac{(BHCO_3)}{(H_2CO_3)}$. Throughout a considerable range the points fall about a straight line, indicating as in the case of mammalian blood that the buffer value is constant. The value of β is given by the slope of this line and is 11 volumes per cent (or 0.49 milliequivalents per liter).

It will be shown subsequently that the plasma of *Urechis* possesses little or no buffer value. Is the total buffer value of the *Urechis* blood adequately accounted for by the quantity of hemoglobin in the corpuscles? In this specimen of blood the oxygen capacity was 3.9 volumes per cent. The buffer value per equivalent of hemoglobin is given

by $\beta/3.9 = 2.82$. This value is intermediate between the buffer values of oxygenated and reduced hemoglobin as it occurs in the cells of the blood of man and of the crocodile, the extreme values being 2.4 for

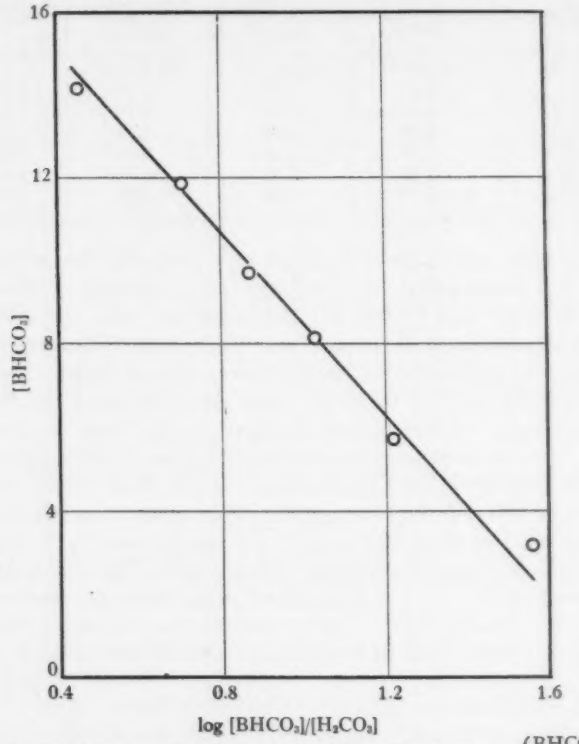

FIG. 5. Relation of combined carbonic acid ($BHCO_3$), to log $\frac{(BHCO_3)}{(H_2CO_3)}$ for blood of *Urechis caupo*. Temperature 19° C. For data see Table VII.

reduced crocodile blood at 29° and 3.47 for oxygenated blood of this species (Dill and Edwards, 1931). The concentration of hemoglobin appears sufficient to account for the total buffering effect of *Urechis* blood.

The effect of oxygenation and reduction upon the carbon dioxide-combining power of blood is considered to be directly related to the reciprocal effect of carbon dioxide (or hydrogen ion concentration) upon the oxygen dissociation constant of hemoglobin (Henderson, 1928. Chapter IV). We have shown in the case of *Urechis* that the latter

is uninfluenced by the quantity of carbon dioxide present, and it is consequently interesting to inquire whether reduced blood possesses the same carbon dioxide-combining power as oxygenated blood. Dill and Edwards (1931) state that both effects are absent or nearly so in the blood of the skate, *Raia oscillata.*

As the effect is proportional to the concentration of hemoglobin, it may be expected to be small in any case. In order to facilitate the experiments, the blood of about ten animals was mixed and the corpuscles separated with the centrifuge. A small quantity of plasma was mixed with the corpuscles, yielding a solution containing 78 per cent red blood corpuscles and having an oxygen capacity of 10.7 volumes per cent. Were the *Urechis* hemoglobin similar to mammalian hemoglobin the reduced solution should combine four or five volumes per cent more carbon dioxide than the oxygenated serum when $\log \frac{(BHCO_3)}{(H_2CO_3)}$ is 0.8. Table VIII shows the result of equilibrating samples of this concentrated blood with air and with hydrogen containing about twenty-two mm. pressure of carbon dioxide. The quantity of oxygen found in the

TABLE VIII

Data on Carbon Dioxide Equilibrium in Oxygenated and Reduced Blood

Oxygen Pressure	Carbon Dioxide Pressure	Carbon Dioxide Combined
mm. Hg	*mm. Hg*	*vol. per cent*
air	21.48	13.42
air	21.50	11.50
air	19.85	10.90
air	23.90	11.21
3.54	23.62	11.85
3.70	23.65	11.50

tonometers used for the " reduced " samples would not oxygenate more than ten per cent of the hemoglobin. Disregarding the first experiment of the series, the combined carbon dioxide is the same within the limits of experimental error in both series.[2] Certainly the phenomenon does not occur with the magnitude commonly observed in the blood of the higher vertebrates, and as in the case of the skate, it may be concluded that the reciprocal effects of oxygen and carbon dioxide upon the equilibrium of *Urechis* hemoglobin with these gases are absent or nearly so.

[2] A second experiment in which the corpuscles were not so highly concentrated and in which the measurements did not agree so closely yielded higher CO_2 values for the oxygenated than for the reduced samples.

The distribution of carbon dioxide between the cells and plasma is of particular interest as *Urechis* is the most primitive type of animal in which the respiratory properties of the corpuscles have been studied. The red blood corpuscle of *Urechis* is a much more "typical" cell than are those of the vertebrates and one looks for properties which contrast it with these more highly specialized erythrocytes.

TABLE IX

Data on Distribution of Carbon Dioxide between Corpuscles and Plasma

Specimen No.	19	22	24	25	26
Temperature of equilibration—° C.	19.5	19.0	20	19	19
Volume of corpuscles—*per cent.*	23.0	34.5	23	34.7	31.7
Low Pressure Experiment					
CO_2 pressure of equilibration—*mm. Hg.*	8.0	12.95	10.50	10.20	9.31
Whole blood-CO_2 content—*vol. per cent.*	8.82	14.20	11.40	11.18	10.80
True plasma-CO_2 content—*vol. per cent.*	7.09	14.15	11.30	10.95	11.45
Separated serum					
CO_2 pressure of equilibration—*mm. Hg.*	53.6	68.4		54.7	47.0
CO_2 content at this pressure—*vol. per cent.*	11.74	19.30		16.54	15.40
High Pressure Experiment					
CO_2 pressure of equilibration—*mm. Hg.*	62.5	62.2		49.5	46.7
Whole blood-CO_2 content—*vol. per cent.*	20.7	23.5		19.4	19.56
True plasma-CO_2 content—*vol. per cent.*	18.9	20.4		17.12	18.82

Several experiments have been made in order to elucidate the respective parts which corpuscles and plasma play in the transport of carbon dioxide, and to determine the extent to which there is an exchange of material between cells and plasma. The procedure has been to equilibrate blood with carbon dioxide at a pressure comparable to that existing *in vivo*. With a part of this solution duplicate determinations were made of the carbon dioxide content of the *whole blood*. The remainder was centrifuged under oil in stoppered tubes. A portion of the plasma so separated was analyzed for the carbon dioxide content of the *true plasma, i.e.*, the plasma in equilibrium with corpuscles at the original carbon dioxide tension. From these measurements together with a measurement of the fraction of the whole blood occupied by the corpuscles, made by hematocrit, the ratio of carbon dioxide concentration in corpuscles and plasma could be calculated. The remainder of the plasma was then equilibrated with a relatively high CO_2 tension in order to give an idea of the buffer action of the *separated plasma*. The foregoing measurements, which are designated as the "low pressure experiment" in the tables, were performed in the mornings on which the blood was drawn. In the afternoon the "high pressure experiment" was carried out. A portion of the whole blood was now

equilibrated at a relatively high CO_2 pressure and its CO_2 content and that of the true plasma determined. The data of the experiments are given in Table IX and certain calculations based on these data appear in Table X. Referring to the latter, several definite conclusions may

TABLE X

Certain Indices of the Distribution of Carbon Dioxide between Corpuscles and Plasma

Specimen No.	19	22	24	25	26
Ratio of CO_2 concentration between corpuscles and plasma					
Low pressure experiments	(2.05)	1.01	1.04	1.03	0.82
High pressure experiments	1.43	1.43	—	1.38	1.29
Change in CO_2 content per unit change in CO_2 pressure					
Separated serum—*vol. per cent per mm. pressure*	0.102	0.093	—	0.125	0.105
True serum—*vol. per cent per mm. pressure*	(0.217)	0.127	—	0.156	0.197
Whole blood—*vol. per cent per mm. pressure*	0.218	0.189	—	0.208	0.234
Total buffer value of blood; β	9.6	7.3		7.6	9.2
Volume of corpuscles—*per cent*	23.0	34.5	23.00	34.7	31.7

be drawn. In other regards the results of the experiments are at variance and the interesting question emerges as to whether the apparent variability in behavior may be due to the properties of the relatively unspecialized cell which serves as erythrocyte in *Urechis*.

The ratio of the CO_2 concentration of whole blood to that in the true plasma is approximately 1.0 in the low pressure experiments. The discordant value in Experiment No. 19 is probably to be attributed to experimental error. This means that CO_2 is about equally distributed between corpuscles and plasma under pressure conditions such as occur in the blood of the worm. The effects of the Donnan equilibrium and the corrections for the volume occupied by solutes in the corpuscles and plasma are neglected and would probably be too small to be significant in measurements as inaccurate as those employed.

The ratio of carbon dioxide concentration between cells and plasma in the high pressure experiments is uniformly greater than one. This result indicates that the principal buffer substances occur within the corpuscle and that the exchange of materials between corpuscles and plasma (the chloride shift) which enables the corpuscles to contribute to the buffer action of the plasma in the higher vertebrates is restricted in the case of the *Urechis* blood corpuscle. It has been shown above that the hemoglobin concentration is sufficient to account for the total buffer action of *Urechis* blood. The apparent restriction in the ex-

change of electrolytes between corpuscles and plasma is perhaps to be related to the tough membrane which may be observed to surround the erythrocyte of *Urechis*.

The relative part played by corpuscles and plasma in *Urechis* blood is further expressed by the estimation of the change in CO_2 content of the components per unit change in CO_2 pressure in passing from the low to the high pressure stages of the experiment. This method of expressing the results is somewhat arbitrary, as the relation is not strictly comparable for varying ranges of pressure. However, for the present purpose of comparing data made at two similar pressures considerably separated, it is convenient.

The increase in CO_2 content with increase of pressure in the case of the *plasma separated at low tensions* is fairly uniform and has an average value of 0.106 volumes per cent per millimeter pressure. This is almost exactly the rate of increase which would be due to the solution of carbon dioxide in the plasma if the absorption coefficient is 0.80 as assumed above. Parsons and Parsons (1923) publish some measurements of the carbon dioxide content of sea water at various CO_2 pressures from which it appears that the rate of increase is 0.109 volumes per cent per millimeter pressure. It is concluded that the plasma of *Urechis* contains at most a negligible quantity of buffer material, the increase in CO_2 content being adequately accounted for by the solubility of carbon dioxide.

Turning to the whole blood, the increase in CO_2 content with change in pressure is reasonably concordant in the various experiments and yields values about twice as great as in the case of the separated plasma. Between the pressures examined the gain in bound CO_2 is about equal to the gain in CO_2 dissolved.[3] This is a further expression of the fact that the corpuscular content is responsible for the buffer action of the blood. The buffer values for the whole blood recorded in Table X are slightly less than that estimated from the experiment recorded in Fig. 5. The variation in buffer value in different samples of blood does not appear to be closely correlated with the volume of cells in the samples. Presumably the variation in hemoglobin concentration in the cells from different specimens indicated in Table III is sufficient to destroy the expected correlation.

The true plasma shows a gain in CO_2 content with increasing CO_2 pressure which is variable but always less than the corresponding gain for whole blood and always greater than the gain shown by plasma separated at low pressures. The latter fact indicates that with increas-

[3] Over a shorter range the gain in bound CO_2 would be relatively greater than this because of the "shape" of the carbon dioxide-combining curve.

ing CO_2 pressure some exchange of material between corpuscles and plasma takes place which increases the ability of the plasma to take up carbon dioxide. This is presumably a "chloride shift" such as occurs in mammalian blood. With the exception of Experiment No. 19 the true plasma always gains less carbon dioxide than does whole blood. This is a further expression of the fact, brought out in the consideration of the ratio of carbon dioxide concentration in cells and plasma, that the exchange of materials affecting buffer action is limited. In Experiment No. 19 the high value of the carbon dioxide uptake of the true plasma is probably due to the experimental error which caused the ratio of carbon dioxide in cells and plasma to appear abnormal.

II. Physiological Observations

The Oxygen Content of the Blood in vivo

Samples of blood were drawn from worms lying in a pan of fresh sea water by inserting into the cœlomic cavity a hypodermic needle attached to a graduated one cc. pipette. The blood flowed into the pipette from its own pressure and was transferred directly into the Van Slyke apparatus for analysis.[4] Immediately following a larger sample of blood was drawn, equilibrated with air for 20 minutes and analyzed, thus yielding a measure of the oxygen capacity of the blood. Table XI

Table XI

The Oxygen Content of Urechis Blood in vivo

Experiment No.	In vivo		Saturated with Air	
	Temperature	Oxygen Content	Temperature	Oxygen Content
	°C.	*volumes per cent*	°C.	*volumes per cent*
9	15	2.87	18	2.85
			18	2.87
20	18.5	3.45	19	3.70
21	18.5	4.11	19.5	4.43
			19.5	4.50

contains the results of three such experiments. The figures are not corrected for dissolved oxygen. In Experiment No. 9 the oxygen content of the blood *in vivo* is equal to that of blood saturated with air.

[4] The pressure existing in the cœlomic fluid may be about sixteen grams per cm.^{2}, for on one occasion in drawing blood from the cœlomic cavity the blood rose in the pipette to a vertical distance of 16 cm. and oscillated about this level as the result of the muscular contractions of the body wall.

In the other two experiments the oxygen content is slightly less than the oxygen capacity. The result indicates that the pressure of oxygen in the blood may be considerably less than that of air. The diminished oxygen content in the blood *in vivo* is largely due to the smaller amount dissolved rather than to incomplete oxygenation of the hemoglobin. Thus, in the case of Experiment No. 21, if we assume the oxygen pressure to be 75 mm., the hemoglobin would be 97 per cent saturated. The combined oxygen would amount to 3.85 volumes per cent if the total combining capacity be taken as 3.96 volumes per cent. The dissolved oxygen would be 0.25 volumes per cent, making the total content of the

TABLE XII

The Carbon Dioxide Content of Urechis Blood in vivo

Specimen No.	In vivo		In vitro		
	Temperature	CO_2 Content	O_2 Content†	CO_2 Pressure	CO_2 Content
	° C.	*vol. per cent*	*vol. per cent*	*mm. Hg*	*vol. per cent*
10	15	7.12	5.62		
11	15.5	8.12	4.65	10.4*	10.67
				36.0*	18.95
12	16.5	8.79	4.4	See Table VII	

* Equilibrated at 18° C.

† Equilibrated with air. Not corrected for dissolved oxygen.

blood 4.10 volumes per cent as observed. It is concluded that the hemoglobin of *Urechis* is almost completely saturated when an abundant supply of oxygenated water is available for respiratory purposes, but that the pressure of oxygen in the blood may be considerably lower than that existing in the water.

The Carbon Dioxide Content of the Blood in vivo

Table XII contains data on the carbon dioxide content of the blood *in vivo* obtained in a manner similar to that of the oxygen capacity. The measurements indicate a normal carbon dioxide content of between seven and nine volumes per cent. Data for the equilibrium of carbon dioxide with the blood used in Experiment No. 12 are recorded in Table VII and Fig. 5. Similar data for two points on the CO_2 dissociation curve of the blood used in Experiment No. 11 are included in Table XII. These data agree closely with those in Table VII. From Table VII it appears that at the CO_2 content observed *in vivo*, 8.79 volumes per cent, the pressure of carbon dioxide would be approximately 7.2 millimeters.

The pH Value of Urechis Blood in vivo

The pH value of blood plasma is given by the equation

$$pH = pK' + \log \frac{(BHCO_3)}{(H_2CO_3)},$$

in which pK′ is a constant dependant upon the properties of the corpuscles and plasma. The value of pK′ for *Urechis* blood is unknown, but it cannot differ greatly from 6.1 when the corrections for temperature (Warburg, 1922), ionic strength (Hastings and Sendroy, 1925) and corpuscular content (Van Slyke, Hastings, Murray and Sendroy, 1925) are taken into account. Since the value of $\log \frac{(BHCO_3)}{(H_2CO_3)}$ for *Urechis* blood at 7.22 mm. is about one, the pH value of the blood plasma *in vivo* must be close to 7.1. The blood is therefore somewhat more acid than human blood and much more acid than sea water. The value of $\log \frac{(BHCO_3)}{(H_2CO_3)}$ indicates that one eleventh of the carbon dioxide of the blood is in solution, the remainder being bound as bicarbonate.

The Exchange of Gas between Blood and Sea Water

Urechis lives in a permanent burrow in flats which are occasionally exposed at low tide. The burrows are U-shaped, having two openings. Water is circulated through the burrow by means of peristaltic contractions of the body wall, which force the fluid backward between the worm and the wall of the tube. The flow thus established serves both for respiration and to bring the animal its food supply. The worms may be kept for long periods in the laboratory confined in artificial burrows constructed of glass tubing, and under these conditions the volume of water circulated and the changes in its gaseous content may be measured. The respiratory and feeding reactions of animals so confined are fully described by Fisher and MacGinitie (1928). They consider that respiration is principally effected by means of water pumped into the hind-gut, through the activity of the muscular cloaca. The structure of the hind-gut as well as the active rhythm through which it is ventilated certainly support this view.

The hind-gut is a large sack extending the length of the body and occupying the greater part of the cœlomic cavity. Its wall is smooth and so thin as to be quite translucent, resembling in this regard a mesenteric membrane. The wall of the hind-gut is bathed directly by the blood, there being no blood vessels (Fisher and MacGinitie, 1928). The peristaltic movements of the body wall must produce some circulation in the blood. Rhythmic contractions of the hind-gut wall appear

to be important in bringing the blood into contact with the hind-gut wall as well as in mixing the water in the hind-gut. If the worm is examined against the light one may see the outlines of the hind-gut, which appears as a relatively transparent region. It may be observed that the hind-gut is the seat of antiperistaltic contractions which sweep over it in the form of deep annular constrictions into which the blood is drawn and carried along. Compared to this effective mechanism the thick, muscular, cuticulated body wall must absorb a relatively small amount of oxygen.

The water within the hind-gut is renewed by a somewhat irregular rhythm. Fresh water is drawn in by a series of from one to upward of thirty small inhalations usually uninterrupted by exhalation. It is then discharged by means of a single exhalation, frequently followed by a period of rest. Fisher and MacGinitie record periods of inhalation lasting from twenty-five to ninety seconds and expirations consuming from ten to fifty seconds.

Samples of hind-gut water have been collected at the moment of expiration. The worm is watched through the glass wall of the aquarium until it begins to discharge the water in a vigorous stream which may be easily observed. At that moment the worm is taken out of the aquarium and the anal end thrust tightly into a funnel terminating under oil in a suitable glass-stoppered bottle. The worm continues to discharge the hind-gut water, which is collected under the oil. From 25 to 35 cc. of water may be obtained at a single discharge. Several discharges were combined to yield material for oxygen analysis by the Winkler method. Carbon dioxide content has been determined with the Van Slyke apparatus. Table XIII contains the results of a number of such determinations, together with control measurements made upon the sea water of the aquarium.

TABLE XIII

Oxygen and Carbon Dioxide Content of Hind-gut Water

Oxygen

Hind-gut water	0.37,	0.37,	0.29,	0.36,	0.36,	0.37	vol. per cent
Aquarium water	0.56,	0.56					vol. per cent

Carbon Dioxide

Hind-gut water	5.26,	5.11	vol. per cent
Aquarium water	4.77,	4.79	vol. per cent

The oxygen content of the expired water is about two-thirds that of the sea water. The partial pressure of oxygen in the hind-gut water is thus about one hundred millimeters and is quite sufficient to account for the high degree of saturation found in the blood *in vivo*.

The carbon dioxide measurements may be evaluated by means of

determinations made by Parsons and Parsons (1923) of the carbon dioxide content of sea water from the Naples aquarium at various pressures. They found at 0.8 mm. CO_2 pressure a content of 4.7 volumes per cent which agrees closely with the values found in our aquaria. Interpolating from their data the carbon dioxide pressure of the expired water corresponds to 4.6 and 6.0 mm. in the two samples examined. Since the partial pressure of CO_2 in the blood is about seven millimeters, a gradient of pressure of about two millimeters occurs across the hind-gut wall.

From the foregoing experiments certain deductions may be drawn relative to the volume of water necessary to "ventilate" the hind-gut. Dr. V. E. Hall, who has been engaged in a study of the respiratory and feeding reactions of *Urechis*, has kindly supplied data concerning the volume of water pumped by *Urechis* through artificial burrows made from glass tubing, and the rate of oxygen consumption of the worms. The average rate of oxygen consumption of two medium-sized *Urechis* was about 0.013 cc. per minute. The amount of water pumped when the worms were not engaged in feeding was about 11 cc. per minute; when feeding, it was about 29 cc. per minute. There is required 2.3 cc. of sea water containing 0.56 volumes per cent oxygen to yield the 0.013 cc. consumed in one minute. When the water is expired from the hind-gut only one-third of the oxygen dissolved in it has been consumed. Consequently 6.9 cc. of water must ventilate the gut each minute. This is about half the amount pumped through the burrows when feeding is not going on. Feeding worms pump about four times the required volume of water, but under these circumstances the water is serving to bring food to the animal as well as for respiration. The size of the animals and their activity are variable and consequently these estimations cannot be very exact. They show, however, that the respiratory activity of the animal is rather nicely adjusted to the metabolic requirements.

The Function of the Hemoglobin of Urechis

The data in Table XI show that the hemoglobin of *Urechis* is almost completely saturated when an abundant supply of aërated water is available to the animals. The preceding considerations indicate that the mechanisms for bringing fresh water into contact with the respiratory surface of the hind-gut operate with a fair margin of safety at each step. Under ordinary conditions it appears that the oxygen bound to the hemoglobin is not utilized and that the oxygen dissolved in the plasma is sufficient for the metabolic requirements. *Urechis* must be added to the list of animals, including *Planorbis* (Leitch, 1916) and *Lumbricus* (Jordan and Schwarz, 1920), in which the hemoglobin does not appear to function if the oxygen supply is adequate.

Light is thrown on the possible value of the hemoglobin to the worms by considering the rate at which oxygen "circulates" through the blood. The problem is somewhat less definite in *Urechis* than in the vertebrates because there are no blood vessels and the ordinary conceptions of arterial, capillary and venous blood do not apply. If we consider 20 cc. to be the blood volume of *Urechis* and four volumes per cent to represent the oxygen capacity, then the oxygen content of the total blood is 0.8 cubic centimeters. Taking the rate of oxygen consumption to be 0.013 cc. per minute, it follows that only one-sixtieth of the oxygen content of the blood is used (and need be replaced) per minute. It is clear from this why the *Urechis* blood is almost completely saturated *in vivo*. It also follows that those properties which assist mammalian blood to give off or take up oxygen and carbon dioxide rapidly during its passage through the capillaries (the reciprocal action of oxygen and carbon dioxide on the equilibrium of these gases with hemoglobin and the transfer of buffer action from cells to plasma) may be dispensed with in *Urechis* blood.

The blood of *Urechis* appears from the foregoing observations to contain a store of oxygen sufficient to last the animal one hour. In addition the hind-gut water itself, having a volume of about thirty cc., contains some 0.11 cc. oxygen. This would serve to supply the metabolic requirement for not more than 8.5 minutes. The total oxygen within the animal consequently is enough to last about seventy minutes.

Consider what would happen if the blood contained no hemoglobin. In it the oxygen concentration would be no greater than in the hind-gut water, say 0.37 volumes per cent. The total volume of oxygen in 20 cc. of blood would be .074 cubic centimeters. At a metabolic rate of 0.013 cc. per minute this would last the animal 5.7 minutes. Adding to this the time which the oxygen in the hind-gut would serve, the total oxygen within an animal without hemoglobin would last about fourteen minutes.

The hemoglobin of *Urechis* consequently extends the period during which the respiratory exchange might be interrupted without depriving the animal of oxygen about five-fold, or for about fifty-five minutes. This is not long enough to carry the animal over the period of a low tide, when the burrows are exposed. It is sufficient to be useful during the "rest periods" which occur after a more or less prolonged period of feeding. According to Fisher and MacGinitie (1928), these rest periods are of two sorts: (1) intermittent periods of from 4.5 to 8.5 minutes separated by about 1.5-minute intervals, during which water is expelled from the respiratory chamber and a new supply taken, (2) a continuous rest of an hour or more during which respiration ceases (or at least is so reduced as to be imperceptible) and no movement of any kind takes place.

The Oxygen Supply When the Tide is Out

On the California coast the tides follow a rhythm in which alternate tides are of unequal height. The low course tides are more nearly equal and in the estuary where *Urechis* was found the flats are not uncovered. During the high course tides the flats are uncovered once a day for a period of six or more hours. During the greatest spring tides the estuary sometimes empties so completely during the lower ebb tides that it does not fill during the succeeding flood tide and in consequence the flats may be bare for 18 hours.

During the period when the tide is out there is available for the worms not only the oxygen in the blood and hind-gut water, which we have seen is adequate for the metabolic requirements for about seventy minutes, but also the oxygen dissolved in the water enclosed in the burrow. An average burrow is about one hundred centimeters long and two centimeters in diameter. It would contain some 314 cc. of water, and if this were saturated with air about 1.76 cc. of oxygen. This would last 135 minutes if used at a rate of 0.013 cc. per minute. The total oxygen supply of *Urechis* during low tide is sufficient for only about three hours according to these calculations.

In order to throw more certain light on the state of affairs during low tide, a series of analyses on the oxygen content of the water in the burrows was made. A rubber tube was thrust down into the burrows and sufficient water for analysis by the Winkler method (70 cc.) drawn out and transferred to a glass-stoppered bottle without exposure to air. A new burrow was selected for each observation. The flats had already become bare when we arrived but had not been so for more than one-half hour to judge from the state of the tide when the first observation was made. The last observations were made from the last burrows to be covered after the greater part of the flat was submerged by the rising tide. The oxygen content of the water left in a puddle by the receding tide, which serves to give an idea of the content of the burrow water before the flat was bared, was 0.34 volumes per cent. This relatively low value may be accounted for by the fact that the observations were made at daybreak. The water had been overnight in an estuary teeming with animal and vegetable life and oxygen losses had not been compensated by photosynthesis. The temperature of the water in the burrows was 15° C. at 6:08 A.M. and had risen to 17° C. at 11:30. At this time the water in the channel was 19° C. The results are recorded in Table XIV.

During the first hour after the flat is bare the oxygen content of the burrow water appears to decrease rapidly and at about the rate expected from the foregoing calculations. There is some irregularity in the

TABLE XIV

Oxygen Content of Water from Urechis Burrows During Low Tide

Time—Samples Collected	Approximate Time Since Flat Became Bare	Oxygen Content of Water	
		From Individual Burrows	Mean
	hours	*cc. per 100 cc.*	*cc. per 100 cc.*
6:08 A.M.	0.5	0.21	0.21
6:24	0.7	0.16	0.16
6:30	0.9	0.13	0.13
6:35	1.0	0.15; 0.11; 0.11	0.12
7:40	2.0	0.24	0.24
7:55	2.2	0.16	0.16
8:00	2.5	0.12	0.12
9:30	4.0	0.06; 0.06; 0.06	0.06
10:30	5.0	0.16; 0.14; 0.14	0.14
11:15	5.7	0.06; 0.16; 0.23; 0.27; 0.27	0.20

individual measurements made during the third hour, but by the fourth hour the oxygen content has definitely sunk to a minimal value of 0.06 volumes per cent. During the fifth hour there is a perfectly definite increase in the oxygen content of the water in almost all of the burrows examined. These measurements support the view that the oxygen in the water inclosed within the burrow and in the blood is insufficient to maintain the normal metabolic rate for the duration of the low tide. After the first hour the oxygen in the burrow water diminishes rather slowly and one must conclude, either that the rate of oxygen consumption by the worm is diminished or that the oxygen in the burrows is replenished by some means. There is reason to believe that both these processes occur. It is well established that the metabolic rate of many marine organisms varies with the oxygen pressure in the environment (Amberson, Meyerson and Scott, 1924; Hall, 1929, and others). The measurements made on the water of the burrows very definitely indicate an increase in the oxygen content during the last hour before the flats were covered. This suggests that the water in the burrows is slowly replaced by the water with which the sand is permeated. The effect is probably related to changes of hydrostatic pressure within the flat occasioned by changes in the tide level, for it is reported that wells in sandy soil near the sea sometimes display definite changes in level related to the tides. The effect becomes noticeable only during the last hour when the tide is rising rapidly. It is presumably occurring throughout the low tide period and serves to check the exhaustion of the oxygen content of the water by the metabolism of the worms. If this view is correct it serves to explain how *Urechis* can withstand the 18 hours of

low water which occur during the spring tides. At these times the small intermediate tide, although unable to cover the flats, will serve to move about the water within the flats and thus replenish to a certain degree the oxygen within the burrows of *Urechis*.

The oxygen content within the burrow never appeared less than 0.06 volumes per cent, which corresponds to an oxygen pressure of about fourteen millimeters. At this pressure the hemoglobin of *Urechis* is nearly 60 per cent saturated. During the greater part of the low tide the pressure of oxygen in the burrow is such that the hemoglobin of the blood will function effectively as an oxygen carrier while very little oxygen will be present in solution in the blood. Provided the oxygen in the burrows does not sink below the observed levels, the hemoglobin of the blood may be expected to transport an adequate supply of this gas to the organs of the body.

Summary

1. The blood of *Urechis caupo* contains hemoglobin enclosed in corpuscles. The oxygen capacity of the blood varies from 2.66 to 7.22 volumes per cent and the percentage of cells in the blood from 18 to 40.

2. The oxygen dissociation curve is measured. Its position does not appear to be influenced by the carbon dioxide pressure. The effect of temperature upon the oxygen dissociation curve is of the direction and order observed in other bloods containing hemoglobins.

3. The carbon dioxide dissociation curve is measured. The ability of the blood to combine with carbon dioxide does not appear to be influenced by the degree of oxygenation of the blood.

4. The buffer value of the blood is 11 volumes per cent and is constant over a considerable range of carbon dioxide pressures. The concentration of hemoglobin accounts for the entire buffer effect.

5. Carbon dioxide is about equally distributed (in concentration) between the corpuscles and plasma. The plasma contains at most a negligible quantity of buffer material. With increased carbon dioxide tension there is a small, but distinctly limited exchange of material between the corpuscles and plasma which increase the ability of the latter to combine with carbonic acid.

6. The hemoglobin *in vivo* is almost completely saturated, but the pressure of oxygen in the blood may be considerably less than that in the surrounding water. The carbon dioxide content *in vivo* is 7 and 9 volumes per cent, corresponding to a carbon dioxide pressure of about seven millimeters Hg. The reaction of the blood is estimated to be about pH 7.1.

7. The "ventilation" of the respiratory organ, the hind-gut, is

considered quantitatively, the result indicating that the respiratory activity is nicely adjusted to the metabolic requirements.

8. The function of hemoglobin and its relation to the oxygen supply during low tide are discussed. It is suggested that the movement of water within the flats due to changing tidal level is important in supplying oxygen when the tide is out.

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OXYGEN AND CARBON DIOXIDE TRANSPORT BY THE BLOOD OF THE URODELE, AMPHIUMA TRIDACTYLA

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This paper is a presentation of the oxygen dissociation curves and of the carbon dioxide absorption curves of the blood of *Amphiuma tridactyla* together with comparisons with similar curves from the literature of the carp, the turtle, the frog, and man. In particular, the applicability of Hill's equation to the bloods of these species and the shape of the oxygen dissociation curve as an adaptive mechanism are briefly discussed. In addition, the properties of the carbon dioxide absorption curves, especially of those which are a result of the low hemoglobin content, are brought out.

Technic

The blood was drawn from the heart 3–5 minutes after the injection of one cc. of 1:1000 heparin in 0.7 per cent sodium chloride to prevent clotting of the blood in the syringe during withdrawal. It was then transferred to a glass container coated with sufficient sodium fluoride and sodium oxalate to make a final concentration of about 0.1 to 0.2 per cent and kept at 2–8° C. until used.

The equilibration of the blood with the desired tensions of oxygen and of carbon dioxide, for 30 minutes at 22–26° C., was performed by a technic similar to that of Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (1922). Blood gases were determined in duplicate by the method of Van Slyke and Neill (1924). Analyses of the gas phase were made by the Haldane-Henderson apparatus. Hematocrite determinations of corpuscular volume were made before and sometimes after samples were drawn for equilibration. No difficulty was encountered in the use of the ferricyanide method for *Amphiuma* blood in contrast with the experience of Krogh and Leitch (1919) with fish blood. Caprylic alcohol as an antifoamer was omitted because of clot formation.

Oxygen Consumption of Amphiuma Blood

The spontaneous diminution of the oxygen of blood so common particularly with nucleated cells is quite marked in *Amphiuma* blood. It was found (Table I) that keeping the blood at 2–5° C. except during

the period of actual equilibration practically eliminated this spontaneous oxygen consumption. The presence of physiological amounts of carbon dioxide apparently diminishes appreciably the oxygen loss. The slight loss which may occur during 20–30 minutes of equilibration and handling is negligible. Wastl (1928) has successfully used KCN to prevent oxygen consumption in the blood of the carp, but its effect in *Amphiuma* blood appeared variable and we abandoned its use in favor of cold to eliminate this source of error.

TABLE I

Effect of Temperature on the Oxygen Consumption of Amphiuma Blood

Temperature = 20° C.

Time *minutes*	Oxygen Content *vol. per cent*
0	2.64
59	1.41
118	0.20
197	0.0

Temperature = 3° C.

Time *minutes*	Oxygen Content at Varying CO_2 Tensions					
	1–3 mm.	25 mm.	42 mm.	44 mm.	53 mm.	60 mm.
0	3.49	4.64	3.00	4.57	4.52	6.23
20			2.98		4.52	6.18
60	3.16	4.70	2.64	4.41	4.46	5.94
120	2.89	4.61	2.55	4.23	4.19	
180	2.69	4.50	2.69	3.99		
240	2.69	4.59				

THE SOLUBILITY COEFFICIENT OF OXYGEN IN AMPHIUMA BLOOD

It is usually assumed as a sufficient approximation that the solubility of a gas in blood or serum, relative to its solubility in water is proportional to the water content of the blood. For *Amphiuma* blood the relative solubilities in serum and in whole blood accordingly would be about 95 per cent and 87 per cent respectively. We have measured αO_2 directly in both serum and whole blood of *Amphiuma* in the following way: Samples of serum and of whole blood were equilibrated with air and with 99.6 per cent oxygen. To diminish spontaneous oxygen consumption the equilibration was performed at 4° C. and the solubility coefficient relative to that of water at the same temperature calculated. After equilibration blood was drawn into a mercury receiver. Sampling and transfer to the Van Slyke apparatus was accomplished by using a Barcroft pipette. Assuming that the oxygen of

the air is sufficient to fully saturate the hemoglobin, αO_2 may be calculated from the difference in the oxygen content of the samples by the equation:

$$\alpha O_2 = \frac{760(V - V')}{P - P'},$$

where V and V' are respective oxygen contents in volume percentage and P and P' are respective oxygen partial pressures in mm. Hg. Table II shows that the relative solubilities of oxygen in *Amphiuma* serum and

TABLE II

Solubility Coefficient of Oxygen in Amphiuma Whole Blood and Serum

Temperature		Oxygen Tension	Oxygen	αO_2
°C. ± .5°		*mm. Hg*	*vol. per cent*	
	3	749	3.90	0.0393
		157	0.84	
	6	754	3.98	
		158	0.79	0.0408
	3	754	3.98	0.0401
Serum				
	3	748	4.04	
		157	0.83	0.0403
	4	157	0.84	0.0410
Av.	4	751	3.97	
		157	0.82	0.0403
		Average αO_2 = 92% of αO_2 water.		
	4	765	12.23	
		161	9.05	0.0400
	4	764	12.28	
		161	9.05	0.0406
Whole blood	4	740	12.90	
		156	9.77	0.0398
Av.	4	756	12.45	
		159	9.29	0.0402
		Average αO_2 = 92% of αO_2 water.		

whole blood are about 92 per cent of that in water; and this figure is used in the subsequent calculations. These figures fail to demonstrate a difference in the αO_2 between serum and whole blood of *Amphiuma*, which is not surprising in view of the low corpuscular volume of the blood.

Hemoglobin Content of the Cells

The corpuscular volume varies considerably and is slightly higher than that found by Southworth and Redfield (1926) for the turtle. The oxygen capacity also varies greatly in *Aphiuma* blood. In Table III it is seen that the ratio of oxygen capacity to cell volume is roughly

Table III

Variation in Oxygen Capacity and in Corpuscular Volume in the Blood of Amphiuma and the Ratio of Oxygen Capacity to Cell Volume

Red Cells *vol. per cent*	Oxygen Capacity *vol. per cent*	Oxygen Capacity *cell volume*
14	2.52	0.18
15	4.52	0.30
17	4.7	0.28
20	5.01	0.25
20	5.8	0.29
25	6.1	0.24
28	8.38	0.30
36	9.64*	0.27
52	9.42*	0.18

* Concentrated blood.

constant, and averages 0.25. The data of Southworth and Redfield (1926) on the turtle show an approximate ratio of oxygen capacity to cell volume of 0.50 and the average figure for human blood is said to be 0.45. The red cells of both *Amphiuma* and the turtle are nucleated and as a rough approximation of the fraction of the volume occupied by the cytoplasm we take 0.80, and calculate the concentration of hemoglobin per unit volume of cytoplasm. It appears from these data that the concentrations of hemoglobin in the cytoplasm of the red cells of *Amphiuma* and the turtle are 0.32 and 0.63 volumes of oxygen per volume of cytoplasm respectively, or 71 per cent and 140 per cent of the concentration of hemoglobin in the cytoplasm of the human red cell, an interesting divergence in this factor for the three different species.

The Oxygen Dissociation Curve

The oxygen-binding properties of both hemoglobin solutions and bloods, except at low and high degrees of saturation, may be represented with sufficient accuracy by the equation of Hill (1910):

$$\frac{HbO_2}{Hb} = K_0 P^n.$$

We have found it convenient to analyze our data on *Amphiuma* blood by means of this equation and it is evident that it holds quite well for this blood as shown by Fig. 1. Here are plotted the points of an

oxygen dissociation curve at 43 ± 3 mm. of carbon dioxide (Table IV) and for comparison, points from similar data for the carp (Wastl, 1928), turtle (Southworth and Redfield, 1926), and man (Bock, Field and Adair, 1924). The respective curves are calculated from the values of

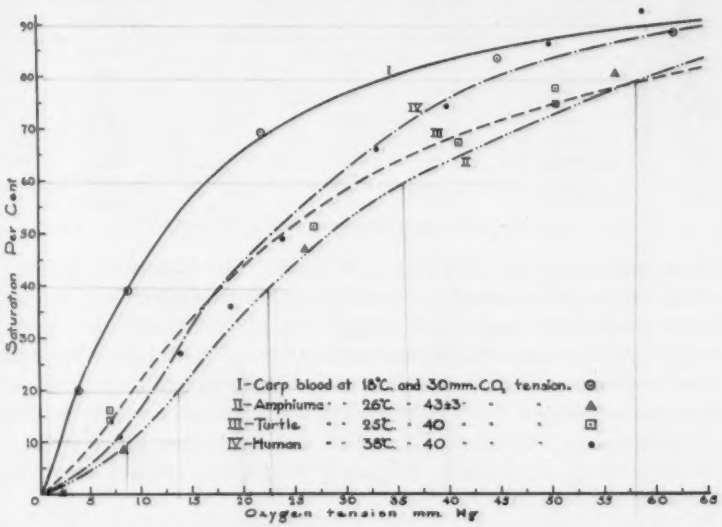
Saturation Per Cent
Oxygen tension mm. Hg
I-Carp blood at 18°C. and 30mm. CO2 tension.
II-Amphiuma · · 26°C. · 43±3 · · ·
III-Turtle · · 25°C. · 40 · · ·
IV-Human · · 38°C. · 40 · · ·

FIG. 1. Oxygen dissociation curves. The lines are calculated by Hill's equation and plotted using the n and K_0 values (Table V) from the original data (points).

n and K_0 for the blood of each species, given in Table V. These constants in turn were obtained in the usual way by plotting log HbO_2/Hb against log PO_2, as in all cases, the points between approximately 10 to 90 per cent saturation fall quite closely on a straight line represented by the equation,

$$\log \frac{HbO_2}{Hb} = \log K_0 + n \log P,$$

from which the constants K_0 and n have been calculated. The conformity of the experimental observations (points) with the equation of Hill (lines) shows that Hill's equation holds reasonably well within the limits specified for these divergent species. *Amphiuma* resembles human blood in showing an "S" shaped oxygen dissociation curve which is absent in the carp and turtle. It is well known that the "S" shaped curve is associated with high values of N (greater than 1.8). This is apparent from the comparison of the curves of these four

TABLE IV

Oxygen Dissociation Curve of *Amphiuma* Blood. Temperature = 26° C. Carbon dioxide tension = 43 ± 3 mm. Oxygen capacity = 8.4 vol. per cent. $\alpha O_2 = 0.92\ \alpha O_2$ (in water).

	O_2 Tension	HbO_2	Saturation
	mm. Hg	*vol. per cent*	*per cent*
1.	2.2	0.01	0.0
2.	8.4	0.70	8.3
3.	15.8	2.2	26.3
4.	26.2	3.9	47.0
5.	56.4	6.7	80.0
6.	79.6	7.6	91.0
7.	143.5	8.4	100.0

forms whose n values vary from 1.4 to 2.0. The absence of the " S " shape has been interpreted by Krogh to signify an adaptation of bottom forms such as the carp to low oxygen tension, whereas the free-swimming forms, *e.g.*, the trout, whose curves are " S " shaped, do not possess this adaptation. The air-breathing *Amphiuma*, despite its habit of remaining submerged for considerable periods, appears not to possess this adaptation. That the " S " shaped curve is not a necessary property of the blood of air-breathing animals is evident from its absence in the turtle, whose n value is 1.5.

TABLE V

Hill's n and K_0 for Carp, Turtle, Man, and Amphiuma Whole Blood

Species	n	$K_0 \times 10^{-3}$	
Carp	1.3	36.0	Wastl (1928)
Amphiuma	1.8	2.5	
Turtle	1.5	8.3	Southworth and Redfield (1926)
Man	2.0	1.9	Bock, Field, and Adair (1924)

EFFECT OF CARBON DIOXIDE ON THE OXYGEN DISSOCIATION CURVE

The increased acidity of the blood with increased carbon dioxide tension has been abundantly shown to decrease the oxygen saturation at a given tension. In other words, K_0 is decreased by increasing carbon dioxide tension. That this effect is manifest in *Amphiuma* is shown by the data of Table VI. The values of K_0 have been calculated by Hill's equation, using $n = 1.8$. This decrease of the affinity of hemoglobin for oxygen with increased acidity is in accord with experience in many species such as man, dog, fishes, and turtle.

TABLE VI

Effect of Carbon Dioxide on K_0 of Amphiuma Blood

	Oxygen Capacity	CO_2 Tension	Oxygen Tension	Saturation	$K_0 10^{-3}$
	vol. per cent	*mm. Hg*	*mm. Hg*	*per cent*	
1.	5.8	*a.* 2.3	53.0	88.0	5.7
		b. 38.0	83.0	84.0	1.8
2.	4.5	*a.* 1.2	26.0	58.0	3.9
		b. 43.5	85.0	87.0	2.2
3.	2.5	*a.* 1.9	44.0	78.0	4.2
		b. 42.7	48.0	62.0	1.5

THE TRANSPORT OF CARBON DIOXIDE

Figure 2, made from the data of Table VII, shows three carbon dioxide absorption curves of oxygenated *Aphiuma* blood. Curve I is characterized by a rather low CO_2 capacity, about 39 volumes per cent at 70 mm. CO_2 tension, and also by the fact that when this blood is equilibrated with gas mixtures lacking carbon dioxide, very little of this gas remains in the blood. Curves II and III, on the other hand, are

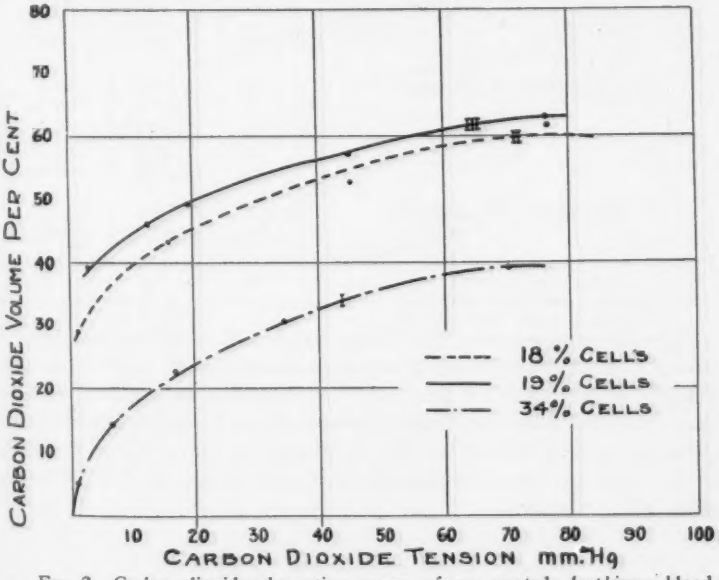

FIG. 2. Carbon dioxide absorption curves of oxygenated *Amphiuma* blood. Temperature 24 ± 2° C.

characterized by a much higher carbon dioxide capacity, approximately 63 volumes per cent at 78 mm. carbon dioxide tension, and further by the fact that when these bloods are exposed to low carbon dioxide tension during equilibration a relatively large amount of carbon dioxide, 30–40 volumes per cent, still remains in the blood. The considerable difference between curve I and curves II and III is probably due to the large variation in hemoglobin content. It is well known that hemoglobin functioning as an acid can combine with only a limited amount of base so that only limited amounts of $NaHCO_3$ will be decomposed into sodium hemoglobinate and carbon dioxide when blood which contains but a limited amount of hemoglobin is exposed to zero or low tension of carbon dioxide.

TABLE VII

Carbon Dioxide Absorption Curves. (See Fig. 4.) Temperature = 24 ± 2° C. Oxygenated blood.

Curve No.	Point No.	CO_2	CO_2
		mm. Hg	*vol. per cent*
I (Cells = 34 per cent)	1.	1.1	4.48
	2.	6.7	14.08
	3.	16.9	22.36
	4.	34.4	30.25
	5.	70.3	38.85
II (Cells = 19 per cent)	1.	3.4	39.45
	2.	12.9	46.11
	3.	19.5	48.79
	4.	45.4	56.78
	5.	76.1	62.94
III (Cells = 18 per cent)	1.	1.1	28.62
	2.	16.3	43.0
	3.	45.5	53.32
	4.	76.4	61.05

The amount of sodium bicarbonate which will be decomposed by the hemoglobin in passing from a definite tension, *e.g.* 40 mm. of carbon dioxide, to a tension of zero may be calculated as follows: let the maximum base bound per unit of hemoglobin be b_0 and the base bound at 40 mm. carbon dioxide per unit of hemoglobin be b. Then if [Hb] be the concentration of hemoglobin in the blood, the $NaHCO_3$ decomposed in the reaction—

$$NaHCO_3 + HHb \rightarrow NaHb + H_2CO_3$$

is obviously $\Delta\ NaHCO_3 = (b_0 - b) \cdot [Hb.]$ A rough extrapolation of the curves of Hastings, Sendroy and Heidelberger (1924) for horse

hemoglobin gives $b_0 = 8$ and $b = 2$. That is, blood in passing from 40 mm. to zero millimeters of carbon dioxide tension will decompose no more than $b_0 - b$ volumes of $NaHCO_3$ for each volume per cent of oxygen capacity. For the *Amphiuma* bloods whose carbon dioxide absorption curves are shown in Fig. 2 the oxygen capacities are respectively 8.5, 4.5 and 4.5 volumes per cent. The corresponding amounts of $NaHCO_3$ decomposable are therefore 51, 27 and 27 volumes per cent. In the case of curve I, however, the amount of $NaHCO_3$ at 40 mm. was 32 volumes per cent which, being less than the maximum of 51, will be entirely decomposed at zero carbon dioxide tension. This was found to be the case as shown in Fig. 2. In the case of curves II and III, where at 40 mm. carbon dioxide tension the $NaHCO_3$ is approximately 55 volumes per cent, only 27 volumes per cent will be decomposed. This was found to be approximately the case as seen in Fig. 2. This calculation, of course, is admittedly the roughest sort of approximation from insufficient data and is offered only as a semi-quantitative explanation of the phenomenon of incomplete decomposition

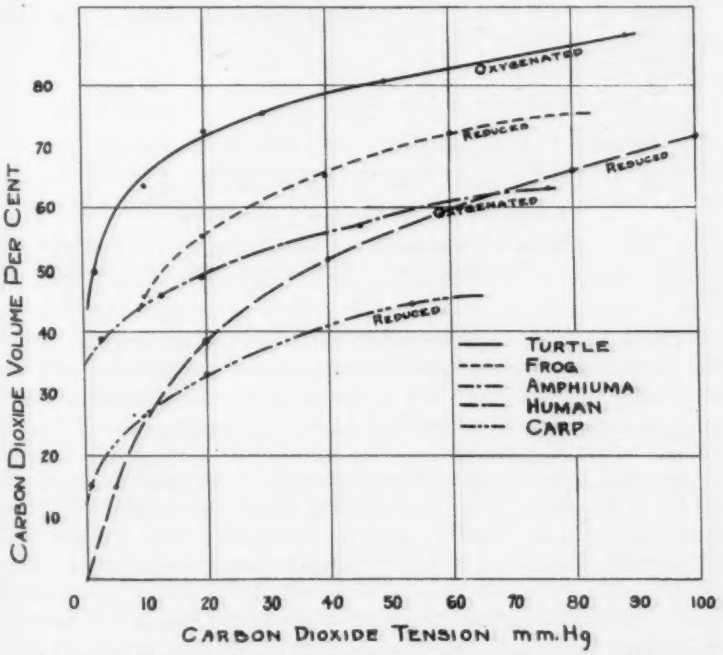

FIG. 3. Carbon dioxide absorption curves of carp, frog, *Amphiuma*, turtle and man. Temperature, human curve 38° C., all others, room temperature.

of $NaHCO_3$ by hemoglobin at zero millimeters of carbon dioxide tension. A quite similar qualitative explanation of the same phenomenon in turtle blood was first offered by Southworth and Redfield (1926).

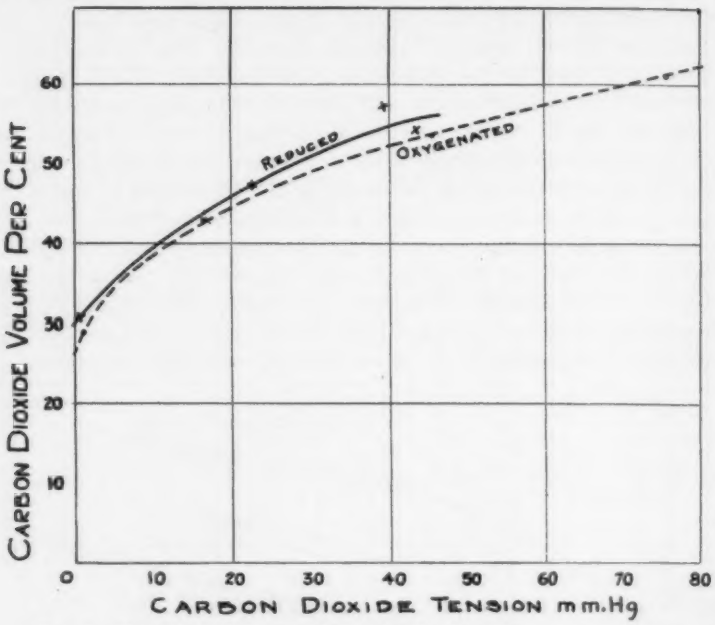

FIG. 4. The effect of oxygenation and reduction on the carbon dioxide absorption curve of *Amphiuma* blood. Eighteen per cent cells. Temperature 24° C.

Figure 3 shows a number of carbon dioxide absorption curves for the carp, frog, *Amphiuma*, turtle, and man, representative of four of the classes of vertebrates. All of the curves for the lower vertebrates resemble each other more than they do the mammalian curve. In these few examples the amphibia occupy an intermediate position with respect to carbon dioxide content between the teleostian carp and the reptilian turtle. The marked flatness of the turtle curve is attributed by Southworth and Redfield (1926) to the low corpuscular volume, *i.e.*, the low Hb of turtle blood. Wastl (1928) gives the same sort of explanation for the essentially parallel curve for carp blood. A similar explanation for the flatness of the *Amphiuma* curve is indicated just as it is for the other lower vertebrates, since the blood of all of these animals shows a corpuscular volume only about one-half to one-third that of human blood.

The Effect of Oxygenation and Reduction of the Blood on the Carbon Dioxide Transport

Figure 4 shows the difference in the amounts of carbon dioxide carried by oxygenated and reduced *Amphiuma* blood. The difference in carbon dioxide content in the two cases is not so great for *Amphiuma* blood as that found by Christiansen, Douglas, and Haldane (1914) for human blood. In fact, this difference amounts to about two volumes

Table VIII

The Effect on Carbon Dioxide Content of Oxygenation and Reduction of Amphiuma Blood. (See Fig. 4.) Temperature = 24° C.

Curve No.	Point No.	CO_2 Tension	CO_2
		mm. Hg	*vol. per cent*
I (Reduced blood, O_2 capacity 4.4 vol. per cent)	1.	0.7	30.87
	2.	22.7	47.28
	3.	39.8	56.94
	4.	43.6	54.34
II(Oxygenated blood, O_2 capacity 4.4 vol. per cent)	1.	1.1	28.62
	2.	16.3	43.0
	3.	45.5	53.32
	4.	76.4	61.05

per cent at physiological levels for *Amphiuma,* while for man the corresponding value is about 5.5 volumes per cent. If the difference in the amounts of carbon dioxide carried by oxygenated and by reduced blood be divided by the oxygen capacity of the sample of blood, a ratio $\frac{\Delta CO_2}{\Delta O_2}$, *i.e.*, the increase of carbon dioxide content per unit of oxygen capacity, is obtained. The value of this ratio in the case of human blood is about 0.28 volume of carbon dioxide per unit volume of oxygen capacity. In five experiments we attempted to determine this ratio closely, but our results were quite divergent. We found for *Amphiuma* blood values of $\frac{\Delta CO_2}{\Delta O_2}$ as follows: 0.23, 0.31, 0.46, 0.71, and 0.93, averaging 0.54. It is obvious that the calculation of this ratio is subject to considerable error since it is the ratio of small differences of large volume; nevertheless, the results are all in the same direction as in the blood of man. The mean value of the ratio for *Amphiuma* is, of course, very approximate. Physiologically, however, oxygenation and reduction have little effect on the transport of carbon dioxide by *Amphiuma* blood. In this respect, also, the *Amphiuma* is like the turtle. The data for the curves of Fig. 4 are included in Table VIII.

SUMMARY

1. The corpuscular volume of *Amphiuma* blood varies considerably, from 12 to 35 per cent.

2. The oxygen capacity varies from 3 to 10 volumes per cent.

3. It is shown that the oxygen dissociation curve is like the typical mammalian curve with certain features in common with those of the turtle and carp.

4. The presence of physiological amounts of carbon dioxide affects the oxygen dissociation curve in the usual way.

5. The comparative values of n and K_0 of the Hill equation are given for the blood of *Amphiuma*, carp, turtle and man, and the equation of Hill for these bloods is shown to hold within the limits specified.

6. The mechanism for the transport of carbon dioxide in the *Amphiuma* blood is much like that in the turtle and the flatness of the carbon dioxide absorption curve is explained as a function of the limited amount of hemoglobin.

7. The difference in carbon dioxide carried by oxygenated and reduced blood is quite small and probably has little physiological significance, though the increase in carbon dioxide content per unit of oxygen capacity is in the same direction as that for man.

The major portion of this work has been carried out under the direction of Dr. William C. Stadie. I wish to thank him for his interest in this problem and for his continual aid and encouragement.

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MOVEMENT AND RESPONSE IN DIFFLUGIA WITH SPECIAL REFERENCE TO THE NATURE OF CYTOPLASMIC CONTRACTION[1]

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INTRODUCTION

It has been repeatedly observed by various investigators that when *Difflugia* and other shelled rhizopods travel, pseudopods, one after another, extend in a given direction, become attached at the tip to the substratum, then shorten and pull the shell forward; but the only reference concerning the mechanism involved in these processes is found in a former paper in which I came to the following conclusions (1926, p. 413): "In this process of locomotion the tip of the attached pseudopod functionally becomes the posterior end. The plasmagel probably changes into plasmasol here and then flows directly into the new pseudopod. . . . The extension of the pseudopods is . . . dependent upon contraction in the plasmagel, resulting in local pressure on the plasmasol." However, in this work but little evidence in support of these conclusions was obtained from observation on *Difflugia*. They were largely based upon the results of detailed observations on the process of locomotion in *Amœba*. Fortunately, I have recently had the opportunity, under very favorable conditions, to make equally detailed observations on the process of locomotion in *Difflugia*. These observations are considered in the following pages.

MATERIAL AND METHODS

Two species of *Difflugia* were used in this investigation: *D. pyriformis* (Leidy) and *D. acuminata* (Leidy). Both were found in the ooze on the bottom of a large permanent pond, the edges of which were frequented by cattle and horses. *Pyriformis* was abundant, *acuminata* rather scarce. The pond is located near Town Hill, Mt. Desert Island, Maine.

All of the specimens studied were well filled with *Chlorella*.

[1] Contribution from the Mt. Desert Island Biological Laboratory. I am greatly indebted to the Director of this laboratory for excellent laboratory facilities and to the Research Corporation for financial aid in procuring assistance in the investigation.

They were very active and they lived well in the laboratory, both in jars and on slides under cover-glasses sealed with vaseline. They were consequently very favorable for making extensive observations on locomotion.

The process of locomotion was studied under Zeiss apochromatic objectives and compensating oculars with magnifications ranging from 200 to 1200 diameters. With the lower magnifications the specimens were observed in watch-glasses, with the higher under cover-glasses supported and sealed with vaseline. In some observations the cover-glass was far enough from the slide so that the specimens could move about freely; in others it pressed on the shells of the difflugiae just enough to prevent locomotion, and in still others it pressed on the shells so much that they broke, some slightly, others considerably. After the shells were broken some of the specimens left and moved about naked. The process of locomotion was thus studied in specimens with shells and in specimens without shells.

The response to tactile and photic stimulation was also briefly studied as indicated below.

Locomotion

Difflugia pyriformis with Shells Free

The shell of *Difflugia pyriformis* is flask-shaped but usually considerably flattened. It consists of a layer of sand grains which vary greatly in size. The interstices between the grains of sand are filled with a yellowish substance which holds them together. According to Leidy, the shells vary greatly in size, ranging from .06 to .58 mm. in length and from .04 to .24 mm. in width. In the specimens studied the shell was about .4 mm. long and .2 mm. wide.

When at rest the living portion of *Difflugia* is usually entirely within the shell and it fills only about three-fourths of the space. When it begins to move a pseudopod forms on the surface of the body below the opening in the shell, then extends out through the opening and advances free into the surrounding medium, until it is about as long as the shell (Fig. 1). As the pseudopod advances it usually swings from side to side freely and extensively but not rapidly. The tip thus frequently moves through an arc of nearly 90° in a little more than one second. In this swinging the tip sooner or later comes in contact with the substratum to which it adheres. Then it contracts slowly and pulls the shell along. Before this pseudopod has disappeared another one usually develops at the opening of the shell and extends at a considerable angle with the old one, but as the old one disappears, the new one becomes directed forward, attaches and then contracts and pulls the shell for-

ward. This process is repeated, one pseudopod developing after another and each pulling the shell forward .2 to .4 mm. Movement of the shell is consequently intermittent.

Sometimes the new pseudopod attaches before the old one detaches

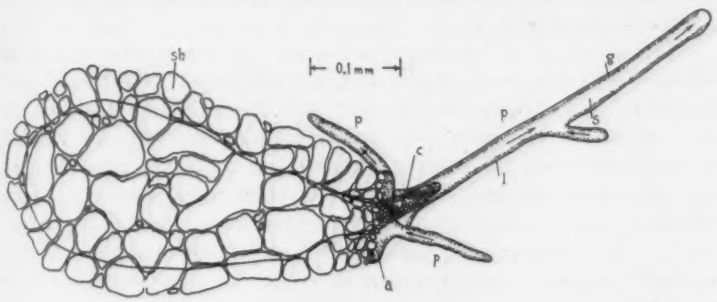

FIG. 1. Camera drawing of *Difflugia pyriformis* in locomotion. *sh*, shell constructed of sand grains cemented together; *p*, pseudopods; *i*, plasmalemma; *g*, plasmagel; *s*, plasmasol; arrows, direction of flow in the plasmasol; *c*, *Chlorella*; *a*, disc of cytoplasm projecting to the edge of the shell; *mm.*, scale showing magnification. The stippling of the plasmagel in this and the following figures is not intended to represent structure. The plasmagel contains numerous small granules, but the plasmasol contains an equal number of the same description.

The large region almost entirely within the shell, enclosed by the solid line, contained so many chlorellae that it was dense green. This region was covered with a thin layer of hyaline substance. The pseudopods usually contain but few chlorellae. At the mouth of the shell the hyaline layer (*h*) usually extends out over the edge of the shell forming a sort of cushion.

Note that the plasmasol extends to the tip of the pseudopod and that there is no hyaline cap.

and occasionally two pseudopods extend simultaneously, become attached and contract simultaneously (Fig. 2*C*). Pseudopods also sometimes branch at the tip or near it and elsewhere. When a pseudopod is extending or advancing there is rapid streaming forward in the middle. This becomes slower and slower toward the surface and disappears entirely before the surface is reached, *i.e.*, there is a layer immediately below the surface which does not move forward. This layer is usually very thin and it is evidently relatively solid, *i.e.*, gel. It forms a tube through which the more fluid part, the plasmasol, flows. At the tip of the pseudopod, the plasmasol stream spreads out and as it comes in contact with the end of the plasmagel tube it gelates, *i.e.*, it is here transformed into plasmagel. The plasmagel tube is consequently built forward by gelation of plasmasol at the tip of the pseudopod just as it is in *Amœba*. But the plasmagel tube is usually open at the tip of the pseudopod and the plasmasol flows forward to the end with nothing corresponding to the hyaline cap in *Amœba* (Fig. 1).

The plasmagel and the plasmasol in the pseudopod contain innumerable small granules and after the pseudopod is fully extended, a group of chlorellae usually appear in the plasmasol at the base. These are, however, never carried to the tip where the plasmasol is transformed into plasmagel, and they consequently never get into and become a part of the plasmagel (Fig. 1).

The surface of the pseudopod is covered with a very thin membrane which is in fairly close contact with the plasmagel. This membrane cannot be seen directly, but the fact that the plasmasol flows to the very tip of the pseudopod and stops there, and the fact that hyaline blisters form in various regions on the surface, as will be demonstrated presently, show that there is a surface membrane, a plasmalemma. This doubtless slides over the plasmagel and stretches as the pseudopod extends. The pseudopod in *Difflugia* is therefore point for point perceptually the same in structure and in the process of extension as it is in *Amœba*.

Nothing could be directly ascertained concerning the mechanism involved in the extension of pseudopods in *Difflugia*, but the fact that they are not in contact with the substratum when they advance shows that they are pushed out by contraction of the portion of the body in the shell. This contraction is doubtless in the plasmagel, just as it is in *Amœba*.

The bending of the pseudopod from side to side is doubtless due to local contraction of the plasmagel on the side of the pseudopod toward which it bends. This contention is strongly supported by the results obtained in observations on response to contact, presented in a succeeding section of this paper.

As soon as the tip of the pseudopod comes in contact with the substratum it adheres to it. Then it flattens and spreads over the substratum and as it spreads it attaches. This continues until the attached surface has increased three or four times in width. Thus the tip of the pseudopod becomes very firmly fastened to the substratum (Fig. 2).

Immediately after the pseudopod has become attached, one or more blisters form at the point of adhesion to the substratum. These consist of droplets of fluid which apparently have been squeezed out of the plasmagel. The fluid aggregates between the outer surface of the plasmagel and the substratum and then spreads laterally (Fig 2*B*). This fluid is definitely differentiated from the surrounding medium, showing that there must be on the pseudopod a surface membrane, a plasmalemma, which has been separated from the plasmagel by the fluid squeezed out. Similar blisters are formed under other conditions as indicated below.

When such blisters form, fluid can actually be seen to flow from the plasmagel, which now becomes clearly visible as a thin granular layer. Under certain conditions this granular layer can be seen to break after the blister containing hyaline fluid has been formed (Fig. 2*A*, *B*); then the granular plasmasol flows through and disperses throughout the hyaline fluid. In this fluid there are a few scattered granules which

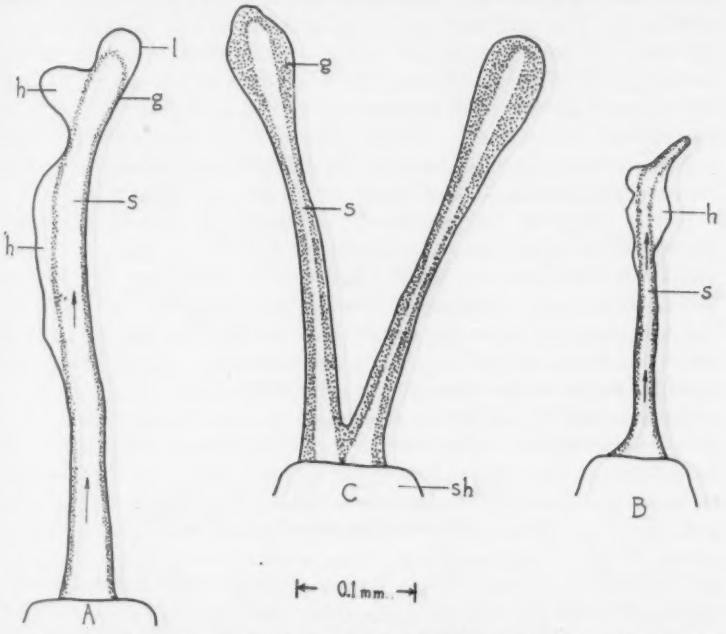

FIG. 2. Camera outlines of pseudopods of *Difflugia pyriformis*, illustrating the process of attachment. *A* and *B*, first stages in attachment; *C*, attachment complete; *h*, hyaline blisters; *s*, plasmasol; *g*, plasmagel; *l*, plasmalemma.

Note that during the process of attachment the hyaline layer in the region that becomes attached increases greatly in thickness and spreads out over the substratum and then gelates.

In the specimen represented in *C* two pseudopods extended and attached simultaneously. This does not often occur. In *C* the pseudopod attached some distance back of the tip.

are in violent Brownian movement. These facts show that the hyaline substance in the blister has liquid properties and that the plasmagel layer is relatively solid. After the blisters have fully formed and have spread over the substratum the Brownian movement in them ceases, indicating that the fluid in them has gelated. This conclusion is supported by other evidence presented below.

Attachment of the pseudopod must be due to adhesive substance on the surface of the plasmalemma, or to adhesive character of the plasmalemma itself. The fluid in the blister formed immediately after the pseudopod becomes attached must be squeezed either out of the plasmagel in the region of adhesion, owing to local contraction, a process resembling syneresis; or out of the plasmasol, owing to local increase in the water permeability of the plasmagel and contraction in this layer elsewhere. The spreading out on the substratum and the flattening of the tip must be due to surface tension which pulls the edges of the pseudopod in all directions over the substratum in the same way that the edges of a drop of oil on water are pulled by surface tension over the surface of the water. If this obtains, the surface tension of the pseudopod-water interface plus that of the pseudopod-substrat interface must be less than the surface tension of the substrat water interface.

After the tip of the pseudopod is attached, the plasmagel becomes thicker and every portion of the pseudopod shortens, resulting in marked and fairly rapid decrease in the length of the pseudopod and in considerable increase in thickness. During the process of shortening of the pseudopod the plasmasol usually does not flow toward the shell as rapidly as the plasmagel retracts, *i.e.*, the plasmasol actually flows forward in relation to the plasmagel, although it is moving toward the shell. Sometimes, however, a branch forms near the tip of a pseudopod while it is contracting and when this occurs the plasmasol usually actually flows forward, *i.e.*, it flows forward in reference to the shell and in reference to points outside the organism. This shows that retraction of a pseudopod in *Difflugia* differs considerably from retraction of a pseudopod in *Amœba*. In the latter, as I have demonstrated elsewhere (1926), the pseudopods shorten, owing to transformation of plasmagel into plasmasol at the tip. In the former they shorten owing to contraction of the plasmagel throughout the entire length. This is, however, eventually followed by transformation of the plasmagel into plasmasol at the tip. Retraction of the contracted pseudopods in *Difflugia* is therefore like the retraction of the pseudopod in *Amœba*.

Sometimes pseudopods are fully extended and then retracted without having become attached. When this occurs retraction is usually much more rapid than it is when they are attached and after they have shortened considerably numerous small blisters appear scattered over the surface, except near the tip; but they become more and more abundant as one approaches the base of the pseudopods. Verworn (1889) observed the formation of similar blisters during retraction of pseudopods in *Difflugia urceolata*. These blisters increase in number as the pseudopods decrease in length (Fig. 3). They are, as will be demonstrated presently, associated with thickening of the plasmagel.

After a pseudopod has become attached, the fluid in the blisters in contact with the substratum gelates and the plasmagel throughout the entire length of the pseudopod thickens. This is, however, much more

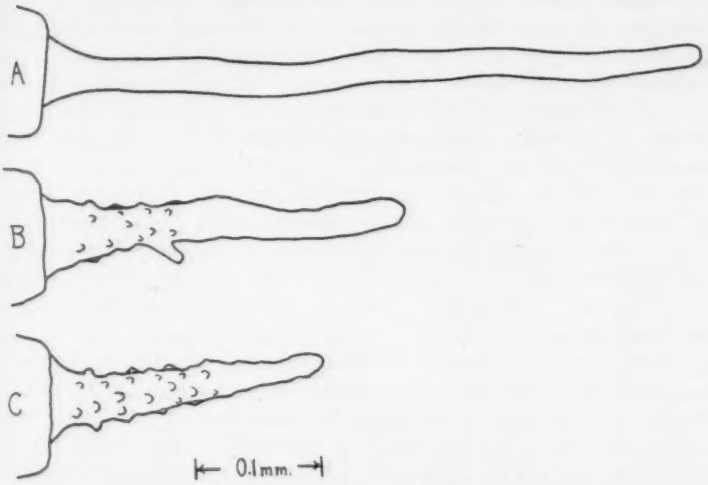

FIG. 3. Camera outlines of pseudopods of *Difflugia pyriformis*, illustrating contraction without attachment to the substratum. *A*, pseudopod fully extended; *B* and *C*, stages in contraction.

Note that during contraction numerous hyaline blisters form on the surface. This is due to localized thickening of the hyaline layer. The thickness of the plasmagel increases markedly when the hyaline blisters form.

evident in specimens in which the shell is firmly held by the pressure of the cover-glass than it is in those in which the shell is free. I shall consider this matter more fully in the following section.

Difflugia pyriformis with Shells Fastened to the Substratum

The shell of *Difflugia* was fastened and held as follows: Several specimens which were nearly the same in size were mounted in water under a cover-glass supported with a ridge of vaseline. Then water was slowly removed until the cover-glass pressed upon the shells just enough to prevent locomotion but not enough to break the shells. In specimens in this condition all the processes of locomotion observed in specimens with the shells free occur except movement of the shell, but there are some illuminating modifications in some of the processes.

Extension, bending and attachment of pseudopods are in all respects precisely the same under the two conditions, but the result of contraction

is very different, the shell moving forward under the one condition and the attachment of the pseudopod breaking under the other.

After the pseudopod has become attached in specimens with the cover-glass resting on the shell, the plasmasol gradually becomes narrower and streaming in it, slower; the plasmagel becomes thicker, and the entire pseudopod some little distance back of the point of attachment becomes thinner. The portion of the pseudopod between the point of attachment and the shell becomes perfectly straight and numerous lines running parallel with the longitudinal axis of the pseudopod appear in the plasmagel, especially near and in the region of attachment (Fig. 4). All this indicates marked strain, owing to violent contraction of the plasmagel especially in the proximal region of the pseudopod. This continues with increasing force until the attachment gives way. This sometimes takes place suddenly. If it does the pseudopod shortens so rapidly and so extensively, after the attachment has been broken, that the distal end actually snaps back to a point not more than half as far from the shell as it was. This demonstrates conclusively that the pseudopod was under rather violent strain before the attachment broke. Usually the attachment of the pseudopod breaks gradually. When this obtains, one point after another, here and there throughout the entire attached portion of the pseudopod, gives way and owing to this the regions of the plasmagel at the tip, which do not give way, are drawn out in strands of considerable length. Finally the attachment of these also breaks, but they retain their form for some time, giving the tip of the pseudopod a distinctly fibrous, brush-like appearance. These strands gradually retract, but the distal end of the pseudopod remains very irregular in outline and much flattened until it disappears (Fig. 4 *C, D, E, F*).

The facts that much of the substance in the attached portion of the pseudopod is drawn out in strands, that these strands contract rapidly and extensively after attachment to the substratum breaks, that the anterior surface of the pseudopod retains an irregular contour until it is withdrawn, and that the anterior end of the pseudopod remains much flattened after it is free, demonstrate conclusively that this substance is a fairly firm, highly elastic gel. These facts and others presented indicate that contact induces gelation, resulting in increase in thickness of the plasmagel throughout the entire length of the pseudopod, and that this causes increase in the elastic strength of the plasmagel, resulting in contraction in the pseudopod and expansion elsewhere. But why does the substance contract after it gelates? This is obviously the central problem concerning the processes involved. It is easy enough to understand why and how an elastic substance which has been stretched con-

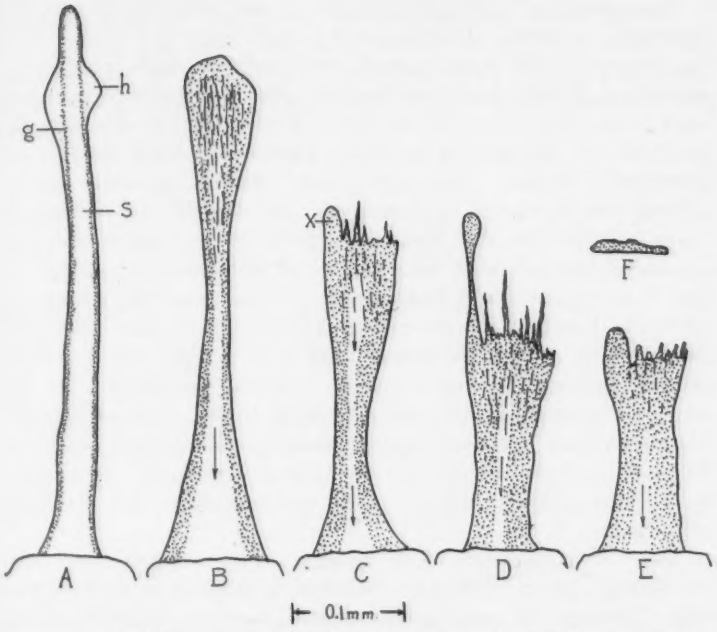

FIG. 4. A series of camera sketches of a pseudopod of *Difflugia pyriformis* representing different stages in the process of contraction in a specimen with the shell fastened to the substratum. *A*, early stage in the attachment of the pseudopod to the substratum; *B*, pseudopod firmly attached at the tip and beginning to contract elsewhere. Note that the plasmagel has increased in thickness and that it appears to be fibrous at the tip. *C*, *D*, and *E*, stages in the contraction of the pseudopod. Note that as the pseudopod contracts, and the attachment breaks, the cytoplasm at the tip of the pseudopod is drawn out into irregular strands which usually shorten considerably after they become detached, indicating that the cytoplasm here is viscous and highly elastic. This was particularly evident in the projection labeled *x*. The tip of this projection remained attached to the slide for some time after the rest of the tip of the pseudopod became free. This resulted in great stretching of the projection. Finally the attachment broke, whereupon the projection contracted very rapidly and extensively as indicated. *F*, outline representing the cross section of the pseudopod near the distal end. Note that the pseudopod was much flattened.

tracts but why and how a sol which has gelated and has not been stretched contracts is difficult to understand. I shall consider this problem presently.

Difflugia pyriformis without Shells

Difflugia without a shell is, as far as my experience goes, never found in nature. Sometimes it will, however, leave its shell if the shell is slightly broken, as the following observations indicate.

Six specimens varying considerably in size were mounted under a cover-glass supported and sealed with a small ridge of vaseline. Then the cover-glass was gently pressed down until the shells were broken, some very slightly, others considerably, after which observations were made from time to time for six days. During the first day there was practically no movement in any of the specimens in which the shell was considerably broken. They were much rounded and there was no indication of formation of pseudopods, but they did not disintegrate. Those in which the shell was only slightly broken behaved normally, *i.e.*, pseudopods extended, became attached and contracted precisely as they do in specimens with intact shells. On the second day pseudopods appeared from time to time on various surfaces in those with badly broken shells. These pseudopods were at first short, but later they extended practically as far as they do in normal specimens. The formation of these pseudopods was not related to the mouth of the shell. They apparently developed equally readily on all surfaces, extending here and there through crevices in the broken shell. They often appeared alternately on opposite sides, one extending while the other contracted.

Later in the day three of the specimens left the shells. One, however, carried with it the edge of the mouth of the shell in the form of a ring. Through this pseudopods extended, one after another, became attached at the tip and contracted, pulling the body along just as in normal specimens. No pseudopods formed elsewhere on the body. In this specimen it could be clearly seen that the plasmasol in the contracting pseudopod flowed directly into an extending pseudopod which developed from the base of the contracting pseudopod as a branch. There was, however, no such violent and rapid shortening of pseudopods as was sometimes seen in normal specimens, especially when stimulated, as will be shown later. The shell therefore seems to function in this. Moreover, the shell serves to coördinate movement in that it confines the formation of pseudopods to one region on the surface of the body; namely, that opposite the opening in the shell. This becomes evident if the movement of normal specimens or that of the specimen with the ring, just described, is compared with that of the specimens which were entirely naked.

In these specimens after they had left the shells just as before they had left it, the formation of pseudopods was not restricted to one surface. As a matter of fact, successive pseudopods rarely formed in the same region of the body; indeed they often formed on diametrically opposite surfaces. This resulted in movement, now in one direction and

then in another; movement which in comparison with that of normal specimens was very irregular in direction and quite uncoördinated.

For some time after these specimens left the shells the pseudopods extended, attached and contracted, pulling the body along, much as they do in normal specimens. Sometimes two pseudopods appeared on op-

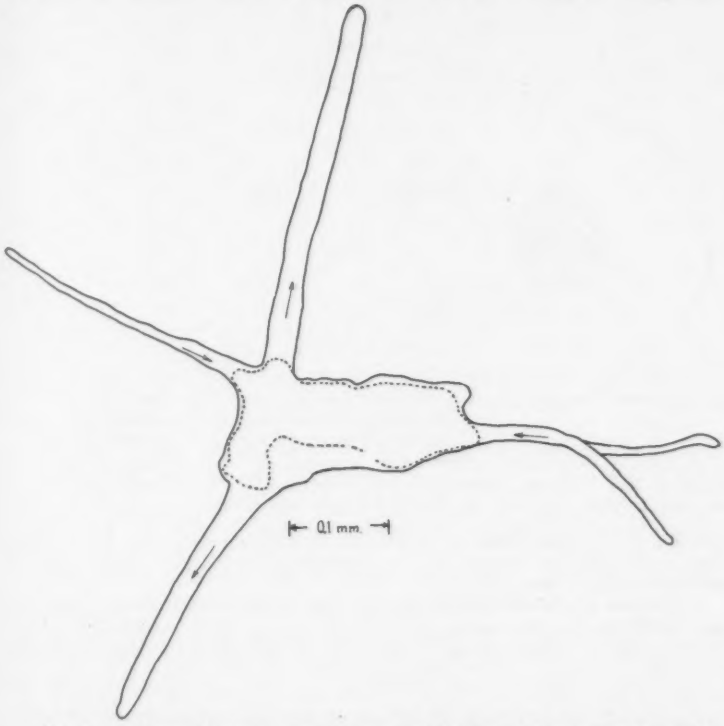

FIG. 5. Camera sketch of a specimen of *Difflugia pyriformis* two days after it had left its shell which had been broken by the pressure of the cover-glass. In this specimen two pseudopods which extended in opposite directions were several times seen to attach simultaneously and then to contract. This resulted in great elongation of the specimen. The portion enclosed by the broken line was well filled with chlorellae; the rest contained numerous small grayish granules.

posite sides of the body simultaneously; then extended, attached and contracted, pulling the body out in opposite directions and greatly elongating it (Fig. 5). The following day all of the specimens were out of the shells and there were several small ones. These were probably fragments which had separated from the large ones. A large

granular nucleus and several contractile vacuoles could now be clearly seen in each of the large specimens (Fig. 6), but none was found in the small ones.

The small specimens moved about in a fairly coördinated fashion (Fig. 7) and some of the large ones now moved much more consistently in a given direction than they did on the preceding day, and in these the

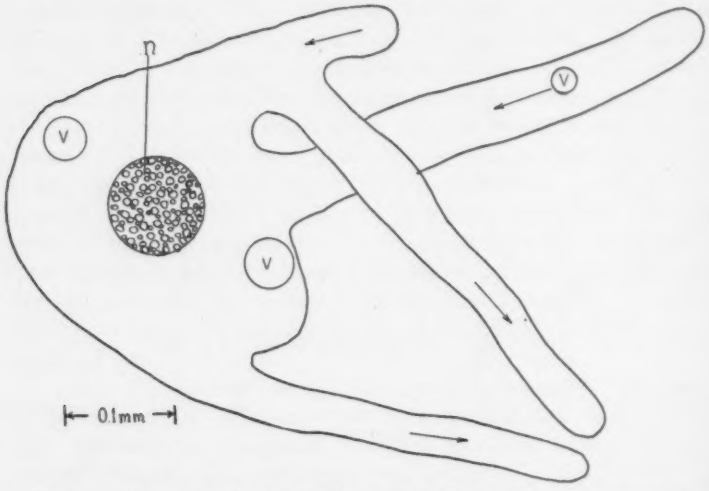

FIG. 6. Camera sketch of a specimen of *Difflugia pyriformis* several days after it had left its broken shell. *n*, nucleus; *v*, contractile vacuole; arrows, direction of flow in the plasmasol.

This specimen moved about fairly consistently and the process of locomotion was essentially the same as that in *Amœba proteus*. It contained many chlorellae, but they were scattered, making it possible to see the nucleus and the contractile vacuoles.

process of locomotion was in all essentials like that in *Amœba proteus* (Mast, 1926). Attachment of the pseudopods at the tip followed by contraction had practically disappeared. A pseudopod advanced in a given direction for a time, then stopped but did not contract. In the meantime another appeared near its base, advanced in the same general direction for a time and stopped, etc., just as in *Amœba proteus*.

The movement of the plasmasol in the pseudopods could be very distinctly seen in these specimens, owing to the fact that the chlorellae were carried out in the pseudopods very much farther than they were in normal specimens. Sometimes they were carried to the tip of the pseudopods, but they never were caught in the gelation of plasmasol at

the tip and consequently never became a part of the plasmagel. This resulted in a very definite differentiation between the plasmasol and the plasmagel, the former being green and the latter greyish.

The following day movement continued in the same way but the specimens were less active, and 24 hours later all of the specimens were

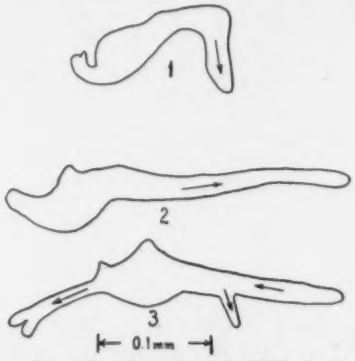

FIG. 7. Camera outlines of a fragment of *Difflugia pyriformis* produced by cutting off a pseudopod from a normal specimen. Interval between successive outlines, 2 minutes; arrows, direction of streaming.

Note that the fragment changed rapidly in form and that relatively large pseudopods developed. This fragment moved about freely and the process of locomotion was in full accord with that in *Amœba proteus*. The fragment contained a surface membrane (plasmalemma), a thin gel layer under this (plasmagel), and a central fluid mass (plasmasol).

much rounded and there was but little movement. All of the specimens lived a few days more, *i.e.*, they lived without shells nearly a week.

These observations were repeated twice with essentially the same results. The fact that the process of locomotion in naked specimens of *Difflugia* is like that in *Amœba proteus* strongly supports the contention that in normal specimens the principles involved are fundamentally the same as in *Amœba*.

Difflugia acuminata

Difflugia acuminata is very much like *Difflugia pyriformis* in structure, the only marked difference being a protuberance on the base of the shell; but it is much smaller, the specimens studied being only about .17 mm. in length and .1 mm. in width.

The process of locomotion is essentially the same in the two species. In both, pseudopods one after another form, extend, attach and contract, pulling the shell along in steps. In both the plasmagel in the pseudopod is in the form of a tube open at the end, and the plasmasol flows out

through this and gelates at the distal end, thus building the gel tube forward and extending the pseudopod. In both, when the pseudopod contracts, the plasmasol flows back through the plasmagel tube, but in *D. acuminata* the backward flow is much more regular than in *D. pyriformis* and it almost invariably flows directly into a new pseudopod which usually extends as the old one contracts (Fig. 8), while in *D. pyriformis* it usually flows back into the body and from there out into a new pseudopod.

Locomotion in *D. acuminata* is essentially in accord with the description of locomotion in *Difflugia sp.* presented in a preceding paper (Mast, 1926, p. 413). It resembles locomotion in *Amœba proteus* with the exception of the rather violent contraction of the pseudopod after attachment at the tip, resulting in marked periodicity in the rate of movement. There is, however, also a tendency toward periodicity in the rate of movement in *Amœba proteus* which was clearly demonstrated by Schwitalla (1924), and it may be that the processes involved in producing this periodicity are fundamentally the same in both forms, *i.e.*, that there is a certain amount of contraction in the pseudopod after attachment in *Amœba* as well as in *Difflugia*.

FIG. 8. Camera outline of *Difflugia acuminata*, illustrating the flow of plasmasol from the old pseudopod directly into the new one.

RESPONSE TO LIGHT

Difflugia pyriformis is definitely positive to light. It does not orient, at least not precisely, but in dishes left in front of a window it aggregates on the more highly illuminated side. This was repeatedly observed in the culture dishes, but it was more evident in a series of tests made by evenly distributing specimens in watch glasses in front of a north window, leaving them for a time and then ascertaining the distribution.

The results obtained in several tests are essentially the same. In one test consisting of five watch-glasses containing a total of 131 individuals there were at the end of 12 hours 103 in the window-half of the dishes and only 29 in the opposite half.

Concerning the process of aggregation I have no information. Rapid increase in illumination probably causes cessation in streaming, but this response if it actually does occur is far less definite than it is in *Amœba proteus*. In naked specimens it was observed that locomotion is much more rapid in low illumination than it is in high, but rapid increase in illumination did not result in sudden cessation in streaming.

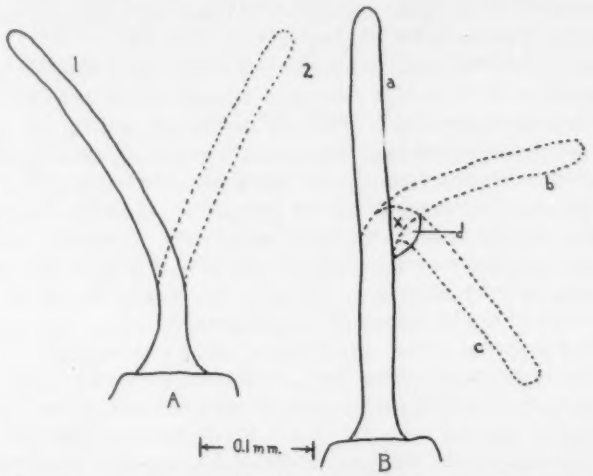

FIG. 9. Camera outline of *Difflugia pyriformis*, illustrating bending of pseudopods. *A*, bending without stimulation; *B*, bending after light mechanical stimulation at *x*; 1, original position of pseudopod; 2, position 1.5 seconds later; *a*, position of pseudopod before stimulation; *b* and *c*, positions one and two seconds later respectively; *d*, blister formed on the side of the pseudopod at the point stimulated.

RESPONSE TO CONTACT

Response to contact was studied as follows: Several specimens of *Difflugia pyriformis* were put into a watch-glass under a dissecting binocular and left until they became active. Then with a glass needle pseudopods in different stages of development were touched in various ways and the effects noted. The results of numerous observations may be summarized as follows:

If the needle is gently brought into contact with the side of a pseudopod, the pseudopod at the point touched almost immediately bends sharply and fairly rapidly toward the side touched. The tip, in this response, frequently swings through more than ninety degrees in less than two seconds (Fig. 9). This bending is obviously due to contraction

of the plasmagel in the region stimulated. Here, immediately after the needle comes in contact with the surface, there is formed a small blister. In a preceding section we have noted that the formation of blisters is associated with thickening of the plasmagel, *i.e.*, with gelation of plasmasol adjoining the plasmagel in the region where the blister appears. If this is true, it is evident that contact causes thickening of the plasmagel, owing to gelation of adjoining plasmasol and that this causes local increase in the elastic strength of the plasmagel and contraction in this region resulting in the bending of the pseudopod.

If, in place of bringing the needle gently in contact with one spot on the pseudopod, it is in close succession brought rather violently in contact with several spots fairly uniformly distributed over the surface, the entire pseudopod contracts, and this usually continues until the pseudopod has been drawn entirely into the shell. The contraction of the pseudopod is sometimes gradual, the pseudopod gradually receding into the shell, but it is usually very rapid, as a matter of fact, so rapid that the pseudopod jerks back into the shell much as a tubicolous annelid jerks into its tube when it is violently stimulated. Verworn (1889, 1914) obtained similar responses in *Difflugia urceolata*.

The withdrawal of the pseudopod is often retarded by a mass of cytoplasm in the mouth of the shell, from which the pseudopod projects. This mass of cytoplasm forms a sort of stopper; it fills the mouth of the shell and projects over the edge in the form of a flange (Fig. 1). When this obtains, the pseudopod, after it is stimulated, shortens, then holds its position until the flange gives way, after which the whole mass, pseudopod and all, suddenly darts into the shell.

The fact that this whole mass of cytoplasm is thus suddenly drawn into the shell shows that stimulation of the pseudopod causes marked contraction in that portion of the body which is located in the neck of the shell and that the body of the organism is fastened to the shell in the basal region; for if this portion of the body were not fastened, it would be drawn forward in place of the portion in the neck of the shell being drawn backward.

When a pseudopod, owing to stimulation, contracts as described above, numerous small hyaline blisters form on the surface. This, as previously demonstrated, is associated with thickening of the plasmagel, owing to gelation of the adjoining plasmasol, and this in turn produces increase in the elastic strength of the thickened plasmagel, resulting in contraction. The fact that stimulation of the pseudopod causes not only gelation and contraction of the region stimulated but also of certain portions of the body within the shell, *i.e.*, in a region some distance from the location of the stimulus, shows that localized contact stimulation pro-

duces in *Difflugia* something which is transmitted through the cytoplasm and then causes gelation of plasmasol followed by contraction. That is, it produces something which is akin to what in higher forms is known as an impulse. This conclusion is supported by results obtained by Verworn in observations on the effect of localized mechanical stimulation of *Difflugia urceolata*. He (1889, 1914) maintains that local stimulation of a pseudopod causes, under certain conditions, contraction of pseudopods which were not stimulated. There is, however, no evidence indicating that this obtains for all rhizopods. Verworn was not able to find it in other species of *Difflugia* and it probably does not occur in *Amœba* (Mast, 1932).

Discussion

I have demonstrated in the preceding pages that the more important factors involved in locomotion and response in *Difflugia* consist of solation of the plasmagel in one region of the body and gelation of the plasmasol in another, of attachment of the tip of the pseudopod to the substratum, of gelation of the plasmasol in the pseudopod correlated with contact and of contraction of the pseudopod due to increase in the thickness and in the elastic strength of the plasmagel in it.

All of these factors are probably also involved in the process of locomotion and response in *Amœba*, but gelation associated with contact followed by contraction of the pseudopod, which plays such an important rôle in the process of locomotion in *Difflugia*, is of little if any significance in the process of locomotion in *Amœba*. To account for locomotion in *Difflugia*, it is necessary then to explain not only gelation and solation but also contraction of the substance after it has gelated. Concerning the processes involved in this, I have no suggestions to offer except that the fact that fluid is squeezed out during gelation and contraction indicates that the processes involved are in some respects similar to those associated with syneresis as found in the gelation of various inanimate substances.

Summary

1. The fleshy part of *Difflugia pyriformis* and *Difflugia acuminata* is in structure much like *Amœba proteus*. There is a thin elastic surface membrane (plasmalemma), a central fluid mass (plasmasol), containing a large granular nucleus, and a relatively solid layer (plasmagel) which surrounds the plasmasol. Probably there is also a hyaline fluid layer between the plasmagel and the plasmalemma but this, if it is present, is much thinner and less conspicuous than in *Amœba*.

2. Locomotion in *Difflugia* is normally brought about by the extension of pseudopods, one after another, and attachment to the sub-

stratum at the tip, followed by contraction which pulls the shell containing the body forward. Movement is consequently intermittent.

3. The elastic strength of the plasmagel is lowest at the tip of the pseudopods. This results in contraction of the plasmagel elsewhere, and this contraction forces the plasmasol out through the plasmagel tube, causing expansion at the tip of the pseudopod. The plasmasol which is in contact with the distal edge of the plasmagel tube continuously gelates and this results in extension of the tube.

4. After the tip of the pseudopod becomes attached, the plasmasol in the tip gelates and the plasmagel throughout the entire pseudopod thickens greatly owing to gelation of adjoining plasmasol. This increases the elastic strength of the plasmagel in the entire pseudopod until it becomes greater here than elsewhere, after which it contracts and the pseudopod becomes shorter and thicker and the plasmasol in it is forced back into the body of the organism.

5. The extension of pseudopods in *Difflugia* is in principle the same as in *Amœba proteus*, and contraction is probably also the same in principle, but it is much more pronounced and much more highly specialized in *Difflugia* than in *Amœba*, in which it does not function appreciably in the process of locomotion.

6. The pseudopods in *Difflugia* after they are extended wave about considerably. This is doubtless due to unequal local contraction of the plasmagel on opposite sides.

7. If the shell of *Difflugia pyriformis* is broken, it leaves the shell in the course of a day or so and moves about fairly freely. Specimens out of the shell sometimes live for a week or more under a cover-glass supported and sealed with a ridge of vaseline. After they have been out of the shell for some time the process of locomotion in such specimens is in all respects like that in *Amœba*, although it is usually much more irregular in direction. Contraction of extended pseudopods, so conspicuous in the process of locomotion in specimens containing shells, has practically disappeared.

8. *Difflugia pyriformis* aggregates on the more illuminated side of dishes in moderate illumination. It does not orient precisely. It is less active in high illumination than in low, especially when it is out of the shell. It may be that this functions in the aggregation observed. Rapid increase in illumination probably causes decrease in the rate of streaming, but this, if it occurs, is much less marked than it is in *Amœba proteus*.

9. Weak local contact stimulation of an extended pseudopod causes sharp bending in this region toward the side stimulated. Strong general contact stimulation causes rapid contraction of the entire pseudopod.

The bending is due to local thickening of the plasmagel in the region stimulated. Contraction of the entire pseudopod is due to thickening of the plasmagel in the entire pseudopod.

10. Contact stimulation results, under certain conditions, in gelation which extends far beyond the region stimulated. There is therefore in *Difflugia* transmission of something akin to an impulse in higher forms.

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THE EFFECT OF DILUTION OF SEA WATER ON THE ACTIVITY AND LONGEVITY OF CERTAIN MARINE CERCARIÆ, WITH DESCRIPTIONS OF TWO NEW SPECIES

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Introduction

The present investigation was undertaken to secure data bearing on the question of the origin, distribution, and evolution of present groups of digenetic trematodes. The specific problem under consideration develops from the observation that several families of the digenetic trematodes have representatives in both marine and fresh-water hosts. A brief statement of the problem was given by Stunkard (1930). If the trematodes found in both marine and fresh-water hosts and assigned to common families, and even to common genera, have true phylogenetic relationships; *i.e.*, if they have descended from common ancestors, rather than consisting of groups that through convergence show morphological and developmental similarities, their distribution raises an exceedingly difficult biological problem.

There appears to be little doubt but that the parasites in question are actually closely related. Among the gasterostomes, *Bucephalus polymorphus* von Baer, 1826 was described from fresh-water fishes and *Bucephalus haimeanus* Lacaze-Duthiers, 1854 was described from marine fishes. Tennent (1906) traced the life cycle of the latter species, and the recent studies of Woodhead (1929, 1930) have demonstrated the development of two species that occur in fresh-water hosts. The similarity in structure and development between the marine and fresh-water species is so striking that it strongly indicates close relationship.

In the Prosostomata there are a number of families whose members occur in both marine and fresh-water hosts. The family Aspidogastridæ, *e.g.* (see account by Stunkard, 1917), contains species that infest mollusks, fishes and turtles of fresh water, and others that occur abundantly in marine fishes. In this aberrant family, also, the morphological and developmental agreement is too close to be satisfactorily explained on the basis of convergence.

Several other families of the Digenea manifest the same type of

distribution. The family Fasciolidæ (see Stunkard and Alvey, 1930) contains one group of genera which infests the livers of terrestrial herbivores, and another, consisting of *Campula, Lecithodesmus, Orthosplanchnus,* and *Zalophotrema,* which occurs in the livers of various marine mammals. In the family Paramphistomidæ there are a large number of genera, most of which parasitize the hoofed mammals, although one genus, *Chiorchis* (see Stunkard, 1929) occurs in the Atlantic manatees. It is of course possible that the sea cows, frequenting the mouths of rivers, acquire these parasites in fresh water. With few exceptions, members of the Heterophyidæ occur in terrestrial vertebrates and the larvæ develop in fresh-water snails, while *Cryptocotyle lingua* infests the intestine of fish-eating birds and the larvæ develop in the marine snail *Littorina littorea* (see Stunkard, 1930*a*). In the family Pronocephalidæ, several genera have been reported from marine turtles, while one species was described (Stunkard, 1930) from the fresh-water turtle, *Amyda,* and a second has been described by Mackin (1930), from *Pseudemys elegans.* According to Fuhrmann (1928) the family Steringophoridæ (syn. Fellodistomidæ, see Stunkard and Nigrelli, 1930) contains species from the intestine of both marine and fresh-water fishes. Representatives of all of these groups have been studied by the senior author and the results afford cumulative evidence that both marine and fresh-water hosts harbor closely related species of parasites.

Nicoll (1915, 1924) lists other families which have representatives in both marine and fresh-water fishes. Furthermore, there are several genera, *e.g., Azygia* (see Manter, 1926), which have species in both marine and fresh-water hosts. No exhaustive review of the literature is here attempted, but sufficient data have been presented to indicate that many groups of trematodes which manifest marked similarity in structure and development have members, some of which infest marine and others which infest fresh-water hosts. The agreement in morphology and manner of development, recurring so consistently in different groups, can hardly be fortuitous, and the majority of investigators are agreed that these groups are formed by closely related rather than convergent species.

If, as has been postulated, these groups contain closely related species, the question naturally arises as to whether the common ancestral form occurred in marine or fresh-water hosts and how the present distribution was effected. A factor which must be kept in mind throughout the discussion is the complicated life history of these digenetic forms. Typically, sexual multiplication occurs in a vertebrate host, and eggs are produced which pass from the body of the host. From the eggs there emerge aquatic, ciliated larvæ which invade the first intermediate host,

always an invertebrate and usually a mollusk, where asexual multiplication takes place. A second type of aquatic, tailed larvæ leaves the first intermediate host, and these larvæ, either by direct penetration or after encystment on aquatic plants or in the bodies of other intermediate hosts, finally reach the vertebrate host. The free-living, larval stages are extremely delicate, ephemeral, and incapable of any extended migration. Consequently the presence of members of a common group in both marine and fresh-water hosts can only be explained by migration of the hosts or by transfer to new hosts.

The migration of free-living species from a marine to a fresh-water habitat, or the reverse, is largely prevented by the physical, chemical, and biological factors that characterize the two types of environment. It is true that marine species have been cut off in arms of the ocean, *e.g.*, the Caspian and Black Seas, which have subsequently become bodies of fresh water, and some of them have persisted although the number of such species is not large. Among the fishes, the anadromous and catadromous forms make regular migrations from one habitat to the other, but these examples stand as exceptions to the general rule. Fresh water imposes an effective barrier against the migration of Foraminifera, corals, echinoderms, cephalopods, and other groups of invertebrates. The relatively few invertebrates that have transferred from the ocean to fresh water have undergone extensive modifications in form and in life history. The free-swimming larval stages, characteristic of marine types, have almost entirely disappeared.

In an excellent study of this subject Needham (1930) has discussed the factors which prevent the penetration of marine organisms into fresh water. Allee (1923) has shown the effect of differences in temperature, oxygen content, and hydrogen ion concentration on the distribution of littoral invertebrates. Adolph (1925) studied certain physiological distinctions between fresh-water and marine organisms. He found that marine organisms show a much greater toleration for fresh water than fresh-water organisms do for sea water. His observations tend to support the long-accepted belief that organisms migrate from the ocean into fresh water, rather than in the opposite direction. In an interesting and suggestive study Marshall and Smith (1930) have attempted to correlate the composition of the body fluids of marine and fresh-water fishes with renal function and to trace the evolution and migration of these vertebrates on the basis of changes in the structure and activity of the excretory organs. Pantin (1931) has studied the triclad turbellarian *Gunda ulvæ*, which occurs in the estuaries of small streams, and found that these acœlomate worms withstand both fresh and salt water, and that in nature they may be exposed to either extreme

for several hours. In tap water they double their volume and lose 25 per cent of their salt content in an hour. The presence of calcium reduces the rate of swelling and the loss of salts, presumably by reducing permeability. The significance of calcium and its relation to the problem of the migration of animals into fresh water was discussed.

The difficulties of migration from one habitat to the other would be greater in the case of parasitic species like the digenetic trematodes than in free-living forms. Where two or more hosts are involved, and where the transfer to the next host is effected by very delicate, short-lived, larval stages, the initial obstacles to migration are augmented by the difficulties inherent in the completion of the life cycle. For such a parasite to change from one location to another, either both primary and secondary hosts must have made the same migration simultaneously, or the parasite must have changed to new hosts as the migration progressed. Furthermore, and probably of greatest importance, the free-living, aquatic, larval stages must be able to withstand the changed environmental conditions and remain infective.

The migration of primary and secondary hosts has not been extensive in recent times at least. The groups of mollusks, fishes, reptiles, and mammals are clearly separated into marine and fresh-water species and this distinction has persisted with but very little change since earlier geological time. The shells of mollusks and skeletons of vertebrates afford suitable material for fossil formation and the geological history of several of these groups is known. According to Zittel (1913) "By means of analogy with recent species we are able in most cases readily to determine whether fossil forms pertain to land, fresh, brackish, or salt-water species." He stated that "not until the boundary between the Jura and Cretaceous is reached do we find any traces of fresh-water snails. . . . In the Wealden, and Cretaceous generally, both land and fresh-water gastropods are quite abundant; they become highly developed and widely distributed during the Tertiary, attaining, in fact, a differentiation nearly equal to that exhibited by the corresponding recent forms."

Since the vertebrate hosts are more active, wider ranging, and longer lived than the molluscan hosts, it would appear probable that if migration is to be considered as the explanation of present distribution, the vertebrate hosts were the principal migrants and that they were primarily responsible for change of habitat. The paleontology of the turtles (Hay, 1908; Williston, 1914) indicates that the marine turtles and the soft-shelled, fresh-water turtles have been separate, independent groups since the Mesozoic era. Looss (1902) described several genera of pronocephalid trematodes from marine turtles; Stunkard (1930) and Mackin (1930) have described members of the same family from the

fresh-water turtles, *Amyda* and *Pseudemys*. The discovery of related parasites in hosts that have been separated since the Mesozoic would suggest that migration of hosts is not to be accepted as an explanation of these cases at least.

There are also serious objections to the explanation involving transfer to new hosts. While host parasite specificity is not so limited as was formerly believed, and it is well known that many parasites may infest several host species, as a rule the possible host species are closely related. For this hypothesis it is essential also that both old and new hosts live in the same habitat, since otherwise they would never encounter the infective larval stages of the parasite. Consequently, if separation into marine and fresh-water species was effected by the adoption of new hosts, the transfer could occur only in those regions where fresh and salt-water habitats overlap, namely at the mouths of rivers. In the transitional zone of brackish water, with the recurrent increase and decrease of the salt content and pH of the water with the rise and fall of the tide, transfer to new hosts may have caused divergence into definitely marine and fresh-water species.

It is thus possible that both migration of hosts and transfer to new hosts or a combination of the two methods may have been operative in producing present distribution of related species. It may be that the distribution of existing groups of digenetic trematodes is correlated with the origin of these groups and this point should be considered in any treatment of the problem. The present complicated developmental cycles could not have been the original or primitive life histories of these species. It has long been recognized that parasites have been derived from free-living progenitors. Competent investigators agree that the trematodes and cestodes have a turbellarian ancestry. The subject was discussed by Meixner (1926) with the following summary, " Es ergeben sich drei Schlüsse:

" I. Dass die Differenzierung der Trematoden und Cestoden mit dem Auftreten der Wirbeltiere eng verknüpft ist.

" II. Dass die zum Parasitismus auf Evertebraten übergegangenen Vorfahren der Digenea und Cestoden entsprechend der heutigen Beschränkung der primären Larven bereits auf verschiedene Wirtstierklassen spezialisiert waren.

" III. Trematoden und Cestoden sind zwei infolge ihres Parasitierens auf Wirbeltieren insbesonders hinsichtlich des Integumentes der Reifestadien abgeänderte Anhangsgruppen der Rhabdocoela."

Concerning the origin of these groups Reisinger (1928) stated, " Bezeichnend für die Amera ist die in vielen Gruppen vorherrschende Neigung zu parasitärer Lebensweise, vielleicht in Ausnützung einer

besonderen, dem ganzen Unterstamm eigenen, stoffwechselphysiologischen Konstitution, die den einzelnen Gruppen den Ubergang zu intramolekularer Atmung (Glykogenabbau) besonders erleichterte. Die Urheimat der Amera ist zweifellos das Meer; der Ubergang zu terrikoler und parasitischer Lebensweise mag sowohl von dort aus wie auch vom Süsswasser erfolgt sein und erfolgen."

Bresslau and Reisinger (1928) concluded that, "Unter den Rhabdocoelen verdienen die Familien der Graffilliden und Anoplodiiden besonderes Interesse, insofern als von ihnen aus vermutlich die Entwickelung der Trematoden ihren Ausgang genommen hat. Nach ihrer ganzen Organization sind die Monogenea wahrscheinlich von Graffilliden, die Digenea von Anoplodiiden oder anoplodiidenähnlichen Kalyptorhynchiern (Rhabdocoela) herzuleiten. Gut stimmt damit überein, dass gerade diese Familien das Hauptkontingent an Parasiten unter den Strudelwürmer stellen."

Consideration of this subject raises one of the most difficult problems in biology, the origin of intermediate hosts and the digenetic life cycle. The original ancestors of the digenetic trematodes must have become parasites of aquatic animals and the evidence indicates that mollusks were the original hosts. The type of reproduction in these mollusks is problematical. It is well established that parasitism increases reproductive activity, that it leads to new and accessory methods of reproduction, and that asexual multiplication is frequently interpolated between the sexual phases. There may have been a sexually mature, free-living stage after asexual multiplication was developed in the invertebrate host. The appearance of the vertebrate host may be correlated with the evolution of vertebrates and their use of mollusks as food.

Presumably the adoption of the parasitic habit occurred at an extremely remote period and the evolution of parasitic life histories and accompanying transformation of the parasites have proceeded hand in hand with the evolution of their hosts. The parallel evolution of hosts and parasites has been demonstrated by many authors. The presence of related species in both marine and fresh-water hosts may be explained by assuming that the primitive hosts harbored the ancestors of present species, and that the hosts have subsequently separated and differentiated into marine and fresh-water species. Such an explanation would imply that descendants of the original hosts have carried their parasites with them since the separation and, as a result of the ensuing migration and modification, the primary hosts, secondary hosts, and parasitic species have evolved together. It may be contended that this explanation merely pushes the problem further back in the time scale, renders it

more difficult of analysis, and less susceptible of experimental treatment. While to a degree this criticism is valid, the postulate may nevertheless be correct, and there are, moreover, experimental means of investigating the question.

Studies of much modified parasitic species and interpretation of their life histories are greatly facilitated if the life cycle contains free-living larval stages. These stages presumably correspond to ancestral ones, since it is generally true that embryonic and early developmental stages are very conservative and tend to remain unchanged regardless of modifications which may occur in the later development of the animal concerned. Since digenetic trematodes have such free-living larval stages, and since these larvæ are the infective agents, providing for the transfer from one host to another,—an experimental study is possible. Knowledge concerning the effects of environmental changes on these larvæ may have significant value in the interpretation of life cycles and distribution. Since the trematodes have two free-living larval stages in the life cycle, data should be obtained for both the miracidial and cercarial stages. It is often difficult if not impossible to secure miracidia in sufficient numbers for such experiments, while cercariæ can usually be obtained in abundance. It therefore seemed pertinent to make a study of the effect of the dilution of sea water on the activity and longevity of marine cercariæ. A corresponding study, already started, on the effects of diluted sea water on fresh-water cercariæ will give data, which, correlated with those from the present investigation, may aid materially in explaining present distribution of related species in marine and fresh-water hosts.

No matter whether the present distribution is explained through migration of original hosts or transfer to new hosts, the essential factor involved is the ability of the free-swimming larvæ to live and remain infective in the new environment. The ability of these larval stages to function in increasing or decreasing salinity indicates the direction of migration and the original home of the original trematode species. Consequently, the experiments reported in the present paper were undertaken.

Material and Methods

All of the cercariæ used in the investigation were obtained from mollusks of the Woods Hole region, and the experiments were done at the Marine Biological Laboratory during the summer of 1930. Data are given in the tables for the following six species: (1) the cercaria of *Cryptocotyle lingua* from *Littorina littorea*, (2) *Cercariæum lintoni* from *Nassa obsoleta*, (3) *Cercaria quissctensis* from *N. obsoleta*, (4) *C. variglandis* from *N. obsoleta*, (5) *C. parvicaudata* from *L. littorea*, and (6) *C. sensifera* from *Urosalpinx cinereus*.

The snails were isolated in small dishes of sea water to determine those from which cercariæ were emerging. Several of those infected by one and the same species were then placed in a small dish for 10 to 12 hours in order to secure large numbers of recently emerged cercariæ. At the end of this period the snails were removed and the cercariæ transferred to small dishes of sea water, usually 20 to 50 in each dish. The sea water was removed from these dishes and replaced by sea water to which various amounts of tap water had been added. Solutions were made up as follows: (I) undiluted sea water; (II) ¾ sea water, ¼ tap water; (III) ½ sea water, ½ tap water; (IV) ¼ sea water, ¾ tap water; (V) ⅛ sea water, ⅞ tap water; (VI) tap water. The dishes were covered to prevent evaporation and kept at the temperature of the laboratory. In each experiment all of the larvæ were subjected to identical conditions except for the different amounts of tap water in the solutions. The only variable factor, therefore, was the amount of tap water and the results show the effects of increasing dilutions of sea water. Observations were made with a binocular microscope at appropriate intervals and the condition and activity of the larvæ noted.

Cercaria of Cryptocotyle lingua

An abstract of this experiment was reported (Stunkard, 1930*c*).

At first the larvæ are very active and all swim vigorously by rapid lashing of their tails, holding the body motionless in a curved position. Swimming movements cause the cercariæ to rise toward the surface of the water and when swimming is temporarily suspended the larvæ slowly sink. They are positively phototropic and accumulate at the light side of the dish. As the vitality of the larvæ diminishes they become progressively weaker and are unable to rise from the bottom. This is due primarily to exhaustion of the tail muscles. The larvæ then extend and retract their bodies and tails, although since there is no functional acetabulum they can make little forward progress. In the solutions which contain 50 per cent or more of tap water the tails soon begin to swell and lose their motility, and later the body swells. Naturally the swelling is more rapid and greater in the more dilute solutions. Apparently swelling is inhibited so long as the tissues are alive and active. The swelling causes cytolysis of the tails and they soon drop off. The tails are frequently lost after a few hours in all of the solutions. In the tables the following notations are used:

Swimming vigorously ++++
Swimming seldom and feebly +++
Contracting vigorously ++
Contracting feebly +
Dead —

The experiments were repeated four times and the results are in substantial agreement. The following protocol is representative.

Solutions	I	II	III	IV	V	VI
No. of cercariae	25	69	53	70	140	172
Aug. 4 Time						
2:00 P.M.	25	69	53	70	140	172
2:20	all ++++	all ++++	all ++++	all ++++	all ++++	80++ 92+
2:30	all ++++	all ++++	all ++++	all ++++	all ++++	86+ 86−
3:00	all ++++	all ++++	all ++++	68++++ 2+++	50++++ 90+++	1++ 171−
4:00	all ++++	all ++++	all ++++	65++++ 4+++ 1++	140++	
6:00	23++++ 1+++ 1++	60++++ 5+++ 2++ 2−	44++++ 7+++ 1++ 1−	58++++ 11+++ 1++	6++++ 4+++ 32++ 10+ 88−	

Solutions.....	I	II	III	IV	V	VI
No. of cercariæ.....	25	69	53	70	140	172
Aug. 4 Time						
8:00 P. M.	19++++ 3+++ 3++	57++++ 8+++ 2+ 2−	33++++ 18+++ 1++ 1−	56++++ 13+++ 1++	7+++ 35++ or + 97−	
Aug. 5 9:00	8++++ 15+++ 1++ 1−	53++++ 10+++ 6−	15++++ 31+++ 6++ 1−	21++++ 30+++ 7++ 5−	10+ 130−	
11:15	8++++ 14++ 3−	41++++ 20++ 8−	14+++ 36++ 3−	15+++ 50++ 5−	5+ 135−	
1:15	22++ 3−	35++++ 26++ or + 8−	50++ or + 3−	5+++ 60 ++or + 5−	5+ 135−	
3:15	22++ 3−	25+++ or ++ 36++ or + 8−	50++ or + 3−	5+++ 60++ or + 5−	2+ 138−	
Aug. 6 9:00	4+ 21−	5+ 64−	53−	70−	140−	

Analysis of the data shows that Solutions II, III, and IV have only slightly harmful effects as the sea water is diluted. In solutions containing 50 per cent or more of sea water the effect is not significant and, in two of the experiments, after 12 hours in Solution No. II the cercariæ were more active and vigorous than those in undiluted sea water. In Solution No. V, which contained ⅛ sea water, the cercariæ were all on the bottom of the container at the end of 2 hours. They had begun to swell noticeably, some had lost their tails, and those whose tails beat rapidly were unable to rise in the water. It is probable that the larvæ are not infective in this concentration for more than a few minutes. The range between ⅛ and ¼ sea water appears to be the critical zone where the dilution of the sea water exerts a markedly harmful effect on the physiological processes of the larvæ. Freshly emerged cercariæ manifest normal swimming movements for only a few minutes when placed in tap water; at the end of 20 minutes all had settled to the bottom, in 30 minutes about ½ of them showed no sign of life and the others soon succumbed.

Cercariæum lintoni Miller and Northrup, 1926

Solutions were made up as in the previous experiment and the same procedure was followed. Since these larva have no tails they can not swim and their activity is restricted to creeping movements as described by Miller and Northup (1926). The experiment was repeated seven times using 20 recently emerged cercariæ in each dish. In two of the tests distilled water was used instead of tap water and the larvæ lived as long, and in certain of the dishes slightly longer than in those containing the same amount of sea water diluted with tap water. The differences were not great and probably are not significant. The results are similar for all experiments and the following protocol, given on p. 253, is typical.

These results are similar to those obtained for the cercariæ of *C. lingua*. The larvæ show very little normal activity after 15 minutes in tap water and it is apparent that they are not infective in this medium. Two larvæ encysted on the bottom of the dish but it is apparent that encystment in the water is not a usual or normal stage in the life history of the species.

Cercaria quissetensis Miller and Northup, 1926

The experiments were conducted as previously described and repeated eight times, using 20 cercariæ in each dish. In swimming, the

Solutions.....	I	II	III	IV	V	VI
No. of cercariæ.....	20	20	20	20	20	20
Time in Hours						
1	20++	20++	20++	20++	20++ or +	20−
2½	20++	20++	20++	20++	20++ or +	
6	20++	20++	20++	20++	18++ or + 2−	
12	20++	20++	20++	20++	13+ 7−	
24	17++ or + 3+ or −	16++ or + 4+ or −	16++ or + 4+ or −	15++ or + 5+ or −	12+ 8+ or −	
36	15++ or + 5+ or −	14++ or + 6+ or −	15++ or + 5+ or −	12++ or + 8+ or −	7+ 13+ or −	
48	11++ or + 9−	5+ 15+ or −	4++ or + 16+ or −	4++ or + 16+ or −	1+ 19−	
60	3+ 17−	2+ 18+ or −	2+ 18+ or −	1+ 19+ or −	20−	
70	1+ 19−	20−	1+ 2+ or − 17−	1+ 2+ or − 17−	20−	

body assumes a spherical form and the tail lashes vigorously. Records were taken every hour and the following protocol, given on pp. 255 and 256, tabulates the results of one experiment.

In this species the cercariæ lose motility after 15 to 30 minutes in tap water. The tails swell in all the solutions containing 50 per cent or more of tap water and soon become detached. There is a tendency for the cercariæ to encyst after 24 hours in the more concentrated solutions and such larvæ removed from their cysts at the end of 72 hours were alive and active. There were only slight differences between the cercariæ placed in Solutions I and II. During the first half of the experiment the larvæ in Solutions III and IV appeared to be affected more than those in Solutions I and II, but in three of the tests they lived longer than those in undiluted or 75 per cent sea water.

Cercaria variglandis Miller and Northup, 1926

This species is very rare. Miller and Northup found only 3 infested snails among 8,875 individuals of *Nassa* examined, and we found only 2 infected snails. The structure of the cercaria indicates that it is the larva of one of the blood flukes, and it swims in active spurts. Only two experiments were made, but the results, given in the protocol on p. 257, indicate that the larvæ are short-lived and very delicate. The procedure was the same as that previously employed.

As the cercariæ lose motility they become distorted, the furcæ coil up and it is sometimes difficult to determine whether or not they are dead. Soon, however, they turn dark-colored and later they tend to float.

Cercaria parvicaudata n.sp.

Two experiments were made with these larvæ. The procedure was the same as that previously employed, although the observations were not continued until the death of the cercariæ. One of the protocols is given on p. 258 and the other is in essential agreement.

The cercariæ in Solution VI (tap water) were all dead and their bodies much swollen at the end of one hour. At this time all of those in Solutions I, II, and III were swimming intermittently, while those in Solutions IV and V were unable to leave the bottom. They were all lying on the dorsal side, bodies bent as in swimming, with the tails moving. After four or five hours they seemed to be more active although they were unable to leave the bottom of the container. At the end of 25 hours, although they were unable to swim, the larvæ in Solution II were more active than those in sea water, and those in 50 per cent sea water were more active than those in Solution II.

Solutions.....	I	II	III	IV	V	VI
No. of cercariæ.....	20	20	20	20	20	20
Time in Hours						
1	20++++	20++++	20++++	20++++ or +++	20++++ or +++	20−
2	20++++	20++++	20++++	20++++ or +++	3+++ 17++	
4	20++++	20++++	20++++	20++++ or +++	2+++ 18++	
6	20++++	20++++	20++++	18++++ or +++ 2++ (tailless)	2+++ 18++ 8 tailless	
12	19++++ or +++ 1++ (tailless)	18++++ or +++ 2++ (tailless)	8+++ or ++ 12++ 9 tailless	7+++ or ++ 13++ 11 tailless	2+++ 18++ or + 15 tailless	
24	12+++ or ++ 7++ 1 cyst 8 tailless	8+++ or ++ 10++ 2−	4+++ or ++ 13++ or + 2 cysts 1−	5+++ or ++ 15++ or +	14++ or + 6−	

Solutions.....	I	II	III	IV	V	VI
No. of cercariæ.....	20	20	20	20	20	20
Time in Hours						
36	7++ or + 2 cysts 6+ or − 5−	5++ or + 9+ or − 6−	13++ or + 2 cysts 5−	11++ or + 3+ or − 6−	8++ or + 3+ or − 9−	
* 48	5++ 2 cysts 13−	6++ or + 14−	7++ or + 2+ or − 9− 2 cysts	6++ or + 8+ or − 8−	5++ or + 3+ or − 12−	
60	2+ or − 2 cysts 16−	2+ or − 18−	3+ 2 cysts 15−	4+ 1 cyst 15−	20−	
70	2 cysts 18−	20−	2 cysts 18−	1+ 1 cyst 18−		

Solutions.....	I	II	III	IV	V	VI
No. of cercariæ.....	20	20	20	20	20	20
Time in Hours						
1	20++++	20++++	20++++	19++++ 1++	15++++ or +++ 5++	12+ 8−
2	20++++	20++++	19++++ or +++ 1++	17++++ or +++ 3+++ or ++	12+++ 8++	6+ 14−
6	14++++ or +++ 6+++ or ++	17++++ or +++ 3++	15++++ or +++ 5++	11+++ 4++ 5−	6+ 14−	20−
12	4+++ 12++ 4−	7++ 5+ 8−	5++ 3+ 12−	4++ or + 6+ or − 10−	20−	
24	20−	20−	2+ or − 18−	2+ 18−		

Solutions	I	II	III	IV	V	VI
No. of cercariæ	39	65	60	80	90	100
Time in Hours						
1	39++++	65+++++	60+++++	80++	90++	100−
6	39++++	65+++++	60+++++	80++	90++	
	39++++ or +++	65+++++ or +++	60+++++ or +++	80++	38++ 45++ 7−	
25	32++ or + 7−	65++ or +	59++ or + 1−	15++ 55+	15++ 47++ 28−	

Cercaria sensifera n.sp.

Four experiments were performed with these larvæ, and the results of one experiment are tabulated in the following protocol, given on p. 260.

There is a pronounced tendency for these cercariæ to encyst when subjected to unfavorable conditions. The process of encystment is rapid and the cysts are either free or attached to the bottom of the container. Presumably this phenomenon is normal and significant for life history studies of the species.

Cercariæ emerge usually at night or in the early morning and the majority soon encyst. They tend to adhere to any object they touch and numbers stick to the inside of a pipette used to transfer them from one solution to another. After attachment they soon encyst.

Discussion

In an investigation of this character, it is desirable to study as many species as possible and representatives of different taxonomic groups. Unfortunately, information concerning the marine larval trematodes of North America is very meager. Only a random sample of the species has been described. The literature dealing with these larvæ was reviewed by Miller and Northup (1926), who described five species from *Nassa obsoleta* at Woods Hole, Massachusetts. It is significant that only one of the five had previously been reported. Since these were almost the only larval trematodes described from the Woods Hole region, an attempt was made to secure them for the present study. All of the five species described by Miller and Northup were found and three of them in sufficient numbers for the experiments. Of the other three species studied, one was shown by Stunkard (1930*a*) to be the larva of *Cryptocotyle lingua,* while the two remaining species are new to science and are described in a later section of this paper.

For these experiments it is essential that cercariæ be available in large numbers. Since cercariæ secured by crushing parasitized snails are immature and not infective (Stunkard, 1930, 1930*b*), such larvæ do not constitute suitable material, and results obtained from them are probably not significant. Consequently, only normally emerged cercariæ were used. Since several hours are required for the emergence of sufficient numbers, some of the cercariæ had been swimming for ten to twelve hours when the experiments were started. This factor undoubtedly accounts for much of the variation shown in the results. Presumably the most recently emerged cercaria lived the longest.

It is apparent in all species studied that tap water exerts an immediate and harmful effect. None of the cercariæ showed normal

Solutions	I	II	III	IV	V	VI
No. of cercariæ	14	17	19	22	20	20
Time in Hours						
1	14++++	17++++	19++++	18++++ 4 encysted	12+ 8−	20−
3	13++++ 1++ (tailless)	17++++	18++++ 1++ (tailless)	14++++ 2++ 4 encysted 2−	20−	
6	12++++ 1++ 1 encysted	17++++	15++++ 3++ 1 encysted	6+ 6 encysted 10−		
15	6++++ 8 encysted	16++++ 1 encysted	15++++ 3 encysted 1+	2+ 6 encysted 14−		
22	3++ 8 encysted 3−	9++++ 8 encysted	4++ 14 encysted 1+	2+ 6 encysted 14−		
39	8 encysted 6−	11 encysted 6−	15 encysted 4−	6 encysted 18−		

activity for more than a few minutes and most of them died within an hour. The bodies and tails became swollen, the tissues underwent cytolysis with the absorption of water, and death followed shortly. Presumably there was a diffusion of salt from the organisms as water was absorbed and the loss of salt would augment and hasten the deleterious effects produced by the imbibition of water. Obviously these cercariæ can be infective for only a very brief period in tap water and it is doubtful whether they could complete their life cycle in fresh water, even if suitable hosts were available.

Larvæ placed in Solution V, containing seven-eighths tap water and one-eighth sea water, were active for considerable periods of time and some of them were able to perform swimming movements for one to four hours. Certain of them, *e.g., Cercaria parvicaudata,* appear to be deleteriously affected after a short time in this concentration, and later they partially recover. They may continue to live for several hours, although the earlier ill effects are not entirely remedied and it is doubtful whether such larvæ would be infective. They are not infective in the case of *C. lingua* and since the life histories of the other species are unknown, experimental test is impossible.

The experiments show a marked difference in the activity and longevity of cercariæ in Solutions V and IV. Whereas a solution containing one-eighth sea water is definitely harmful, cercariæ manifest little in the way of ill effects in solutions containing 25 per cent sea water. In one-fourth sea water the larvæ live almost as long as in greater concentrations, although they are usually less active after the first few hours. The sluggishness may be due to the increased water content. Cercariæ may succumb somewhat more quickly in one-fourth sea water than in more concentrated solutions, but they are normal in appearance and activity for sufficiently long periods of time to indicate that they may be infective and able to function normally in continuing the life cycle.

Considerable interest attaches to the observation that larvæ are active and apparently normal for almost if not quite as long in solutions containing 50 per cent or more of sea water as they are in undiluted sea water. In certain experiments, cercariæ actually lived longer in one-half than in undiluted sea water, although they were not normally active and probably not infective for longer periods than larvæ in sea water.

The ability of cercariæ to withstand dilution of sea water is roughly proportional to the dilution which occurs in the larger bays. Cowles (1930) reported that, "The salinity of Chesapeake Bay, like that of other long bays and estuaries, gradually decreases, with very few exceptions, from the mouth to the head; and the bay is known as a

brackish body of water, although the failure as a rule, of the fresh waters from the land and the saline waters of the sea to mix completely, and the variation in the volume of fresh and salt water entering the bay, result in different degrees of brackishness. The surface data at the mouth of the bay show a variation in salinity from about 19 to 30 grams per liter, while near Baltimore there is a variation from about 3 to 11 grams per liter." . . . "The bottom salinities recorded on our cruises for the mouth of the bay varied from about 26 to a little over 32, while in the region of Baltimore they varied from about 6 to 17."

It appears more than probable that such transitional zones, extending sometimes for a distance of a hundred miles or more, with ocean water at one end and fresh water at the other, provide the ecological settings in which species become transformed physiologically and structurally from marine to fresh-water organisms, and vice versa. Due to the well-known and constantly appearing variations which occur among animals, certain fresh-water species may have become adapted to life in brackish and eventually sea water, while similarly, marine species may have entered fresh-water habitats.

If the larvæ of parasitic species are able to survive in a new and different environment long enough to find and infect a suitable host, either a former host that has migrated into the location, or a new host species, the life cycle may be completed. The long list of snails which serve as intermediate hosts of *Fasciola hepatica*, under different conditions in various parts of the world, demonstrates the extent to which that species has acquired new hosts and the work of Cort (1918) illustrates the ability of other trematode larvæ to successfully attack new hosts. In the case of a marine or brackish-water species entering fresh water, the essential factor is the ability of the free-swimming larvæ to withstand the hypotonic medium until infection is accomplished, since in the body of the host a medium of higher salt content and tonicity is encountered.

The present experiments record the ability of six marine cercariæ to withstand dilution of sea water and show that these larvæ manifest normal activity for considerable periods of time in solutions containing only one-eighth to one-fourth sea water. The observations indicate that these cercariæ are able to complete their life cycles in brackish water and denote the extent to which marine species may migrate into brackish and fresh water. The paper thus contributes toward the solution of the problem of the distribution of related species in marine and fresh-water habitats.

PLATE I

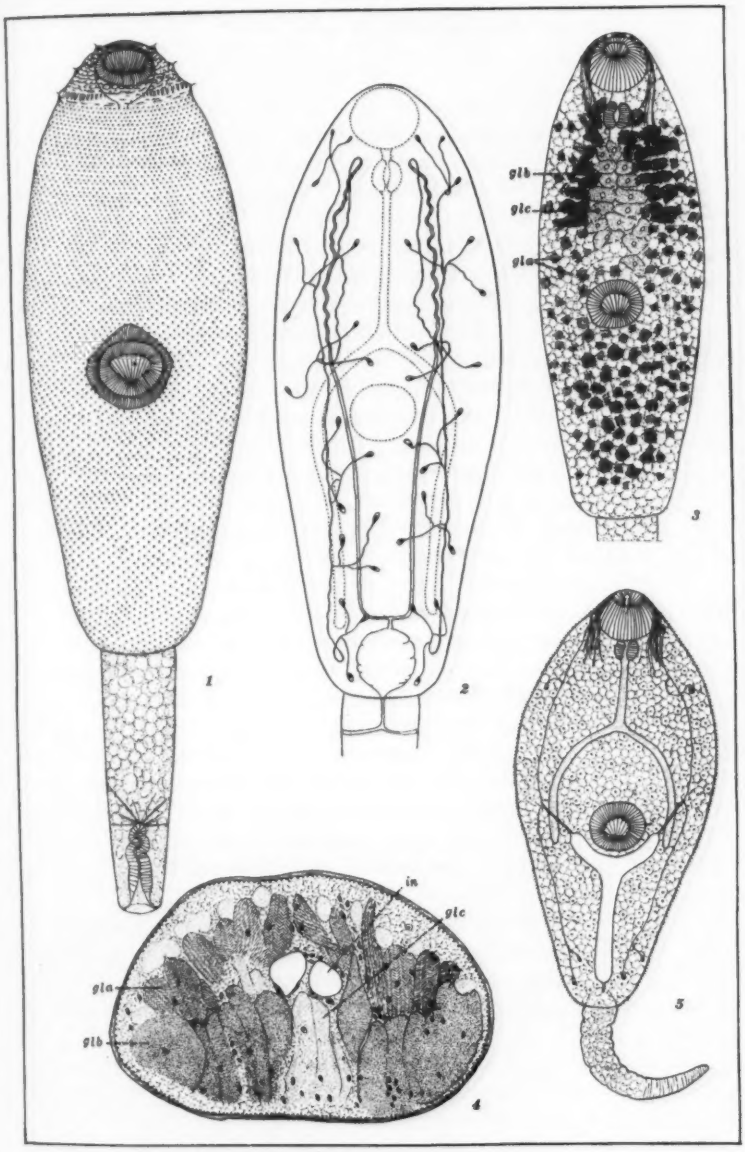

FIG. 1. *C. sensifera*, ventral view.
FIG. 2. *C. sensifera*, excretory system.
FIG. 3. *C. sensifera*, ventral view, showing distribution of gland cells.
FIG. 4. *C. sensifera*, cross section, showing three types of gland cells.
FIG. 5. *C. parvicaudata*, ventral view.

Cercaria parvicaudata n.sp.

(Fig. 5)

This species occurs in about one per cent of the specimens of *Littorina littorea* examined at Woods Hole. The cercariæ are produced in sporocysts which occupy the lymph spaces of the snail. The gonad is the principal seat of infestation and frequently this organ is entirely destroyed.

The cercariæ are small, with oval to pyriform bodies and short tails. The length of the body varies from 0.14 to 0.36 mm. in contracted and extended condition. The tail is very active; it may be contracted to a length of 0.06 mm. or extended until it exceeds the body in length. Ordinarily when the worm is attached or creeping, the tail is contracted and manifests a nervous, twitchy motion. In swimming the body is contracted into a short, wide form, bent ventrally and the tail is extended and lashes violently.

The body is covered with minute spines and the oral sucker bears a stylet 0.015 mm. long by 0.0032 mm. wide. There is a small thickening on the stylet near its tip. The acetabulum is situated near the middle of the ventral surface and measures from 0.03 to 0.05 mm. in diameter.

The cercariæ encyst readily. A portion of a dissected snail was left for six hours in a watch glass and ten cercariæ had encysted among the sporocysts. Other cysts were found in the tissue of the snail which had been fixed and later sectioned. The cysts measure approximately 0.17 mm. in diameter. There are gland cells of two types distributed throughout the parenchyma of the body; one is filled with refractive, spherical granules, the other is slightly opaque and contains very fine granules. On either side of the mouth there are openings of ducts which pass backward and appear to communicate with other glandular cells situated in the preacetabular region, although the connections of these ducts were not determined with certainty. Presumably they are the ducts of penetration or salivary glands.

The oral sucker is spherical to oval, 0.035 to 0.06 mm. in diameter. It is followed almost immediately by a small pharynx and the esophagus extends about one-half of the distance to the acetabulum. The digestive ceca terminate blindly near the level of the caudal margin of the acetabulum.

The excretory vesicle is Y-shaped, with a long stem and short branches. Its wall contains large, deeply-staining cells. Four flame cells have been definitely located and these are shown in the figure. Others were observed, but their connections were not traced. The reproductive organs are represented by a mass of cells which are dorsal and anterior to the acetabulum.

This species belongs in the large and heterogeneous group of Xiphidiocercariæ, but further attempts to relate it must await a more complete knowledge of its morphology or information concerning its adult form.

Cercaria sensifera n.sp.

(Figs. 1–4)

This species has been found only in the oyster drill, *Urosalpinx cinereus*, and it was present in fourteen out of 594 specimens examined during the summer of 1930. Six infestations were found in two hundred and six snails collected at Woods Hole during the first week of April, 1931. The parasites infest the interlobular areas of both the reproductive and digestive glands. In an uninfected snail the visceral mass is plump, the liver is yellow and the gonad is cream-colored, whereas in a parasitized snail the organs are shrunken, the gonad may be destroyed, and the body is lighter in color.

The cercariæ (Fig. 1) are large and clearly visible to the unaided eye. The body is oval in shape, more or less elongated and narrower posteriorly, flattened dorsoventrally, and widest in the preacetabular region. It is truncated posteriorly and the attachment of the tail is terminal. The cercariæ vary considerably in size and manifest much elongation and contraction in locomotion. They are not active swimmers and tend to remain near the bottom of the water. After a time swimming movements alternate with creeping ones. In swimming, the tail is elongated; it does not lash about, but the cercaria moves by undulatory movements of the body and tail. The chief propulsive force comes from the anterior half of the body. It slowly bends ventrally and then snaps backward, pulling the larva forward. The movement is continued through the posterior part of the body and tail producing the sinuous motion of the larva.

With the exception of the anterior end, the body is covered with a thick granular cuticula which bears large, closely set spines. Those in the anterior row are considerably larger than the others. This row is interrupted in the midventral region. There are 44–48 spines on the dorsal side and 10 on each side ventrally. These spines measure 0.005–0.006 mm. in length. This region of the body is sometimes contracted to produce a distinct collar-like effect. There are about forty-five annular rows of spines in the preacetabular region and about 130 to 135 such rows on the body. The spines in successive rows alternate with each other and those around the acetabulum are arranged in concentric rings. The cuticula of the tail is thin and smooth.

The larvæ are bottom forms, and attach readily to any available

surface. When picked up in a pipette they frequently adhere to the inside of the tube and can be dislodged only with great difficulty. They may become attached either by the suckers or by the tip of the tail and after attachment they soon encyst. If placed in solutions that are irritating, *e.g.*, too strong concentrations of vital dyes, they encyst almost immediately. The cyst consists of two layers, a thick, opaque, external covering and a thin, transparent, very tough, inner membranous layer.

Neither of the cyst walls is readily stainable by ordinary dyes. The cyst is oval, flattened on the side of attachment, and measures from 0.2 to 0.23 mm. in width by 0.23 to 0.27 mm. in length. The tail is always detached in encystment and may remain attached for a time to the surface of the cyst. Normally the worm fills the cyst completely. The outer cyst wall is easily removed by rolling a cyst between a slide and cover glass, but it is difficult to get the worm out of the inner membranous covering without injury.

Living cercariæ may extend to a length of 0.9 mm. and contract until the length is no greater than the breadth. The tail also is capable of much extension and contraction; it may be very much shortened or elongated to almost the length of the body. In the latter condition it is slender with an expanded, cup-shaped portion at the end. The caudal tip is usually introverted in a characteristic manner (Fig. 1), although the invaginated portion may be protruded and apparently bears a sticky substance, by means of which the cercariæ adhere to objects. Fixed and stained specimens measure from 0.21 to 0.47 mm. in length and 0.14 to 0.26 mm. in width. In such specimens the tails vary from 0.12 to 0.26 mm. in length. The acetabulum is situated slightly behind the middle of the body. In living specimens it measures from 0.08 to 0.1 mm. in length and from 0.1 to 0.115 mm. in width, while in fixed and stained specimens it measures from 0.68 to 0.76 mm. in diameter.

The anterior end of the body is covered by a smooth, thin, unarmed cuticula which extends backward as far as the caudal margin of the oral sucker. This region bears a number of papillæ, arranged irregularly in two or three rows, and each papilla terminates in a bristle. Similar structures occur around the margin of the acetabulum and presumably they function in a sensory manner. This idea is expressed in the specific name, *sensifera*. The caudal one-fourth or one-fifth of the anterior unarmed area forms a zone which frequently is marked by small longitudinal furrows.

The body is filled with gland cells of several types. In the region between the pharynx and acetabulum there are twelve large unicellular glands. These cells (Figs. 3, 4, *glc*) have very small secretory granules and open to the surface through twelve pores situated at the anterior tip

of the body above the oral sucker. They seem to correspond to penetration glands of other cercariæ and indicate that the larvæ at some later stage bore into the tissues of an intermediate host. The cortical layer of the parenchyma contains numerous dermal glands. In addition, the dorsal half of the body contains numerous gland cells (Figs. 3, 4, *gla*), the cytoplasm of which is filled with bacilliform granules or rods. These cells do not stain with neutral red and in sections counterstained with erythrosin the secretory products appear yellowish. The ventral portion of the body is largely filled with gland cells (Figs. 3, 4, *glb*) whose cytoplasm contains large spherical secretory granules. The cell contents stain intensely with erythrosin. In the anterior half of the body these cells appear to be arranged in four longitudinal fields, separated by the large ventrolateral nerve trunks and the esophagus.

Attempts were made to study the cercariæ in solutions of various vital dyes. With neutral red the spine-covered portion of the cuticula and the cells which secrete it quickly take up the stain and this red or pink layer at the surface of the body masks the action of the stain inside. It is clear, however, that the contents of the digestive ceca assume a deep red color. The stem of the tail, with the exception of the caudal third or fourth, is filled with large fluid globules that take the stain and become a brick-red color. Young specimens do not take the stain at all, and at a later stage the bodies of the larvæ assume a diffuse pink or rose color. The use of other stains, methylene blue, dahlia, pyronin, brilliant cresyl blue, Janus green, light green, and methyl violet did not give significant results on this cercaria. If very dilute solutions were employed the staining was slight and diffuse, not differential, and when stronger solutions were used the cercariæ encysted very quickly.

The mouth opening is subterminal and the oral sucker measures from 0.06 to 0.08 mm. in diameter in living specimens. In fixed and stained specimens the diameter is from 0.05 to 0.06 mm. There is a short prepharynx, the pharynx measures from 0.02 to 0.027 mm. in diameter, and the esophagus is long, extending about two-thirds of the distance to the acetabulum, where it bifurcates to form the intestinal ceca (Fig. 4, *in*). The ceca end blindly about two-thirds of the distance from the acetabulum to the caudal end of the body. The esophagus as well as the ceca is lined with epithelium and this point is significant for life history and taxonomic identification.

The excretory system (Fig. 2) has been worked out in detail and the pattern confirmed on dozens of cercariæ. The system forms as two separate parts, one right and the other left, as described for other cercariæ. The longitudinal ducts fuse near the posterior end of the body and this portion becomes the future excretory vesicle. With the con-

striction that forms the tail the posterior end of the vesicle is denoted and the two excretory pores open on either side of the tail as shown in Fig. 2. The details of the excretory system are shown in the figure. The collecting ducts extend forward to the level between the pharynx and oral sucker, passing on the ventral side of the intestinal ceca. The anterior portions of these ducts contain excretory concretions. Each collecting duct turns posteriad and this recurrent stem contains two large ciliated areas. At the level of the intestinal bifurcation the recurrent ducts divide into anterior and posterior branches. Each of the anterior and posterior branches bears three clusters of flame cells with three cells in each cluster. The cone of cilia in a flame cell measures from 0.008 to 0.01 mm. in length.

The reproductive organs are represented by a mass of deeply staining cells, the anlagen of the gonads, situated in front of the excretory vesicle, and a strand of cells which extends forward connecting with another cell mass in front of the acetabulum. The strand of cells will form the gonoducts and the cell mass in front and sometimes to the left of the acetabulum is the anlage of the genital pore and copulatory organs.

The cercariæ are produced in rediæ. A redia has two " feet," a birth pore near the oral sucker, and an intestine which contains orange-colored granules. In a small redia the germ masses are situated in the caudal third of the body and the posterior tip may be protruded in a tail-like or foot-like protuberance that is used like the feet in locomotion. There is a muscular lip-like snout in front of the oral sucker; the sucker measures from 0.05 to 0.07 mm. in diameter and in a young redia the intestine extends through three-fourths of the body length. Rediæ increase to a length of 2.1 mm. and a width of 0.4 mm. The small rediæ may have one or more fully formed cercariæ in their bodies and large rediæ contain from ten to thirty more or less developed cercariæ.

Cercaria sensifera belongs to the Megalura group of cercariæ, outlined by Cort (1915) and extended by Sewell (1922). It agrees closely with *C. purpuræ* and *C. patellæ*, marine species described by Lebour (1907, 1912) and indeed may be specifically identical with *C. purpuræ*. Slight differences between the present specimens and the account of Lebour in regard to the arrangement of the gland cells, the presence of a " neck " region, and the relations of the excretory system and the tail, make it impossible to determine with certainty whether the specimens may be referred to *C. purpuræ*. Accordingly, a new name is proposed for them with the understanding that it will disappear as a synonym if further studies prove the American species to be identical with the European. In the paper cited, Lebour (1912) predicted that the adult stage of the parasite occurs in a bird. Two years later, Lebour (1914)

compared young stages of *Parorchis acanthus* Nicoll with *C. purpuræ* and identified the cercaria as the larval stage of that species, confirming a prediction made by Nicoll some years previously. Believing that *Parorchis* is closely related to the echinostomes, Lebour stated that the second intermediate host is probably a mollusk. In a later report, Lebour and Elmhirst (1922) reported that *C. purpuræ* encysted in the mantle of *Cardium edule* and *Mytilus edulis*. Their figure of the cyst from side view indicates that it is on the surface rather than embedded in the mantle of the host. The observations of Lebour and Elmhirst appear to complete the life cycle of the species but the experiments were not sufficiently controlled to exclude other possibilities. In the first and only successful experiment reported, the cercariæ used "swam actively by a strong side to side lashing of the tail." It is questionable whether these larvæ were actually *C. purpuræ* since megalurous cercariæ do not employ this method of swimming. Two types of larval trematodes were found encysted in the single specimen of *Cardium* used in the experiment and the more abundant species was identified as *Echinostomum secundum*. Consequently, two types of cercariæ were introduced unless the intermediate host was already infected when the experiment was begun. Apparently the authors did not know whether or not the bivalves used in the experiment were previously infected, and results of such experiments are not at all conclusive. If *C. purpuræ*, like *C. sensifera* will encyst on any surface to which it adheres, it is only natural that the larvæ should encyst on the mantle of mollusks placed in aquaria with them, and it does not necessarily follow that these mollusks are the normal intermediate hosts.

The idea at once presents itself that *C. sensifera* is a larval stage of *Parorchis avitus* Linton, 1914, an unusual trematode in which free miracidia were found in the uterus and in which each miracidium contained a well developed redia. Such a life cycle would explain the observation of Lebour that sporocysts or other preredial stages of *C. purpuræ* were not found in the snail host. Linton (1928) gave a further discussion of *P. avitus* and argued against the suggestion of Nicoll that the American species is identical with *Parorchis acanthus*.

Summary

The problem concerning the origin and distribution of closely related parasites that occur in marine and fresh-water hosts is discussed. Migration of hosts and transfer to new hosts are the only explanations that appear tenable. The essential factor is the ability of the free-swimming larvæ to live and remain infective in a changed environment. Experiments on the ability of six species of marine cercariæ to withstand dilu-

tion of sea water show that these larvæ manifest normal activity for considerable periods of time in solutions containing only one-eighth to one-fourth sea water. The observations indicate that these cercariæ are able to complete their life cycles in brackish water and denote the extent to which these organisms may migrate into brackish or fresh water.

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